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THE UNIVERSITY OF ALBERTA

MEMBRANE ANCHORAGE AND QUATERNARY STRUCTURE OF ALKALINE PHOSPHATASE FROM HUMAN LIVER

by

KENNETH MICHAEL HAWRYLAK

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE

OF MASTER OF SCIENCE

IN

EXPERIMENTAL PATHOLOGY

DEPARTMENT OF PATHOLOGY

EDMONTON, ALBERTA

SPRING, 1989

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The undersigned certify that they have read, and recommend to the faculty of Graduate Studies and Research for acceptance, a thesis entitled MEMBRANE ANCHORAGE AND QUATERNARY STRUCTURE OF ALKALINE PHOSPHATASE FROM HUMAN LIVER submitted by KENNETH MICHAEL HAWRYLAK in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE in EXPERIMENTAL PATHOLOGY.

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Supervisor

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Date: March 23, 1989....

ABSTRACT

Human liver alkaline phosphatase is bound to the plasma membrane by a covalent linkage to phosphatidylinositol, but can be released by proteases or an exogenously added phosphatidylinositol phospholipase C; the released form of the enzyme is dimeric. In contrast, when alkaline phosphatase is solubilized from the plasma membrane by non-ionic detergent, it has a tetrameric molecular weight. Thus, the enzyme may be a tetramer in its native membrane. Since the detergent-solubilized tetramer can be converted to dimers by phosphatidylinositol phospholipase C or proteases, the phosphatidylinositol membrane anchor is essential in order for two dimers to interact together to form a tetramer. Purified tetramer, when run on a sodium dodecyl sulphate polyacrylamide gel system, had only one protein band corresponding to that of the subunit molecular weight of the enzyme, indicating that no other proteins were constituents of the tetramer. Treatment of various hydrophobic forms of alkaline phosphatase with phosphatidylinositol phospholipase C converted them to forms that would not adhere to an octyl-Sepharose column, indicating that phosphatidylinositol is the major exposed hydrophobic constituent of the enzyme. A model of human liver alkaline phosphatase and of its conversions to various forms is presented to explain the aforementioned results.

Also, preliminary evidence is presented that demonstrates an activity in the plasma membranes of human liver that is

capable of releasing alkaline phosphatase into a soluble, hydrophilic form. This release requires both an acidic pH and 1-butanol. These data are consistent with the alkaline phosphatase releasing activity being a phospholipase, of either the C or D type, located on the plasma membrane of human liver cells.

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LIST OF ABBREVIATIONS

DAF	decay accelerating factor
DEAE	N,N'-diethylaminoethyl
EDTA	ethylenediamine tetraacetic acid
HSPG	heparin sulfate proteoglycan
MES	2[N-morpholino]ethanesulfonic acid
mfVSG	variable surface glycoprotein in a membrane form
MOPS	3-[N-morpholino]propanesulfonic acid
NP40	Nonidet P-40
PAGE	polyacrylamide gel electrophoresis
pI	isoelectric point
PI	phosphatidylinositol
PI-PLC	phosphatidylinositol phospholipase C
PMSF	phenylmethylsulfonyl fluoride
SDS	sodium dodecyl sulfate
sVSG	soluble variable surface glycoprotein
VSG	variable surface glycoprotein

CHAPTER ONE

REVIEW OF PREVIOUS WORK

A. PURPOSE OF THIS STUDY

The physiological role(s) of human alkaline phosphatases have not been conclusively identified. Of importance to understanding these role(s) is the physical association of the enzyme to phosphatidylinositol (PI) and how this phospholipid functions to attach alkaline phosphatase to the external surface of the plasma membrane. This enzyme is a very frequently assayed serum enzyme in hospital laboratories, and is the most polymorphic human enzyme known. A genetic deficiency of the enzyme with three degrees of severity is known. Yet, there is much to be learned about this enzyme.

This thesis reports my studies on the novel form of membrane anchorage for human liver alkaline phosphatase and its effect on the quaternary structure of this enzyme. The data reported in this thesis, along with that gathered by others, will hopefully lead to an understanding of the role(s) of alkaline phosphatase under normal physiological conditions. With this knowledge we can get insight into the involvement of the enzyme in states such as hypophosphatasemia, malignancies, and liver disease.

B. HISTORICAL ASPECTS

In the early 1960's, Slein and Logan found that when culture filtrates of Bacillus cereus, free of alkaline phosphatase, were injected intravenously into animals, hyperphosphatasemia resulted (1). The hyperphosphatasemic response was proportional to the amount of culture filtrate injected and the response could be measured one hour after the injection. In the same study, slices of rabbit epiphyseal bone or kidney cortex, or suspensions of rabbit leukocytes, were incubated with small amounts of the culture filtrate. This caused a marked release of alkaline phosphatase from these tissues. However, the factor(s) causing this effect could not be well characterized, since it was not homogeneous. By the use of a N,N'-diethylaminoethyl (DEAE)-cellulose column, Slein and Logan were able to separate from the culture filtrates of B. cereus two peaks of phospholipase activity, one which caused hyperphosphatasemia and one which did not (2). Further characterization of the DEAE column eluate revealed at least three different phospholipases, one of which was active against phosphatidylcholine and phosphatidylethanolamine, another against sphingomyelin, and the third against PI (3). The greatest hyperphosphatasemia factor activity (as measured by alkaline phosphatase release from rabbit kidney cortex homogenate) was associated with the PI phospholipase. They explained the release of alkaline phosphatase as due to damage to the membrane by the hyperphosphatasemia factor.

Eleven years passed before any more work was published to follow up the observation that the hydrolysis of PI and release of alkaline phosphatase from plasma membranes had occurred simultaneously. In 1976, Ikezawa et al. purified to homogeneity the phosphatidylinositol phospholipase C (PI-PLC) of B. cereus, and used it to quantitatively release alkaline phosphatase from rat kidney slices. They proposed that alkaline phosphatase in the plasma membrane of rat kidney cells may be associated with PI (4). This was to be the beginning of a new field of study in which the novel physical association (i.e. linkage) of certain membrane proteins with PI, as evidenced by the release of these proteins from the membrane by PI-PLC, was to get the attention of an ever increasing number of researchers.

C. PHOSPHOLIPASES (GENERAL)

Phospholipases are classified as A_1, A_2, C, or D types, according to the bond cleaved within the phospholipid (Figure 1). The A_1 phospholipases are widely distributed in nature whereas the A_2 phospholipases are found predominantly in snake and bee venoms, and in the mammalian pancreas. The C phospholipases have been isolated predominantly from bacteria while the D phospholipases are found primarily in plants (5).

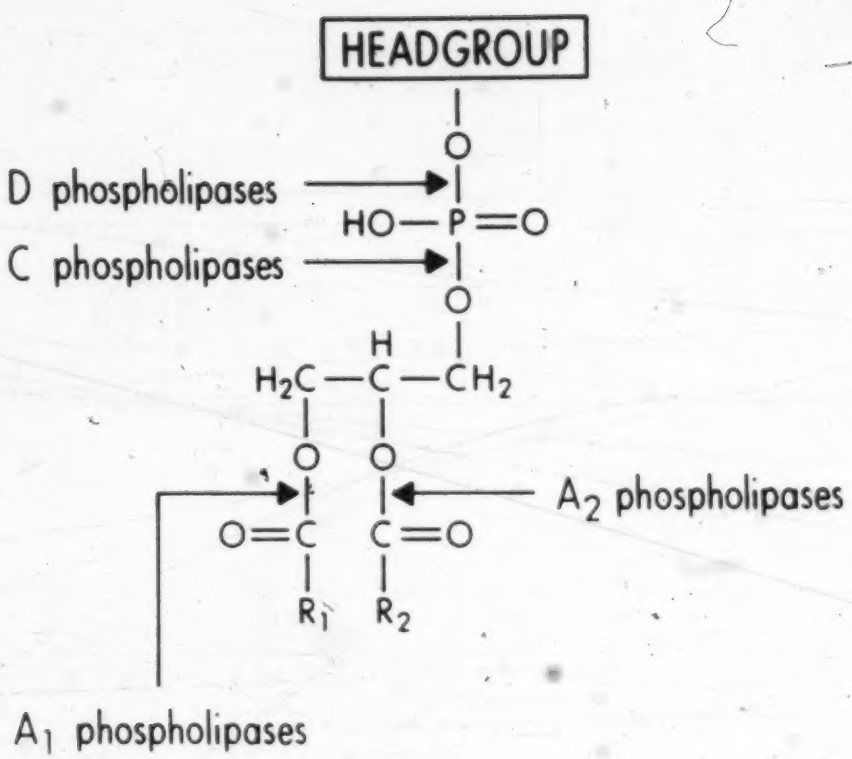


Figure 1. Sites of phospholipid cleavage by the classes of phospholipases.

D. BACTERIAL PHOSPHATIDYLINOSITOL PHOSPHOLIPASE Cs

Bacterial PI-PLCs (1-phosphatidyl-D-*myo*-inositol inositolphosphohydrolase [cyclic-phosphate-forming], EC 3.1.4.10) (6) catalyze the reaction shown in Figure 2. Four PI-PLCs from three different bacterial genera have been characterized to date (Table 1). The PI-PLCs have slightly acidic pI values, are not dependent upon divalent cations for activity (as indicated by the lack of inhibition of activity by chelators such as ethylenediamine tetraacetic acid (EDTA) and *o*-phenanthroline), are of a relatively small molecular weight, and are most likely active in a monomeric form. They are inhibited by cations. Divalent cations in the 10^{-3} to 10^{-2} mol/L range inhibited the enzymes whereas the monovalent cations had to be in the 10^{-1} mol/L range to cause significant inhibition.

Optimal pH for activity was slightly alkaline (pH 7.2--7.5) for the *B. cereus* PI-PLC (4) and was pH 7.5 for the *Bacillus thuringiensis* PI-PLC (9). The *Staphylococcus aureus* PI-PLC had a pH optimum of 4.6 (16). The enzymatic activity of PI-PLCs from *S. aureus* and *B. thuringiensis* were shown to be dependent upon sulfhydryl groups since treatment of these enzymes with compounds such as monoiodoacetic acid or *p*-chloromercuribenzoate resulted in a considerable loss of activity (12,17).

Very recent work on the specificity of the *S. aureus* PI-PLC has shown no activity towards PI-4-phosphate and PI-4,5-bisphosphate although PI itself was hydrolyzed (18).

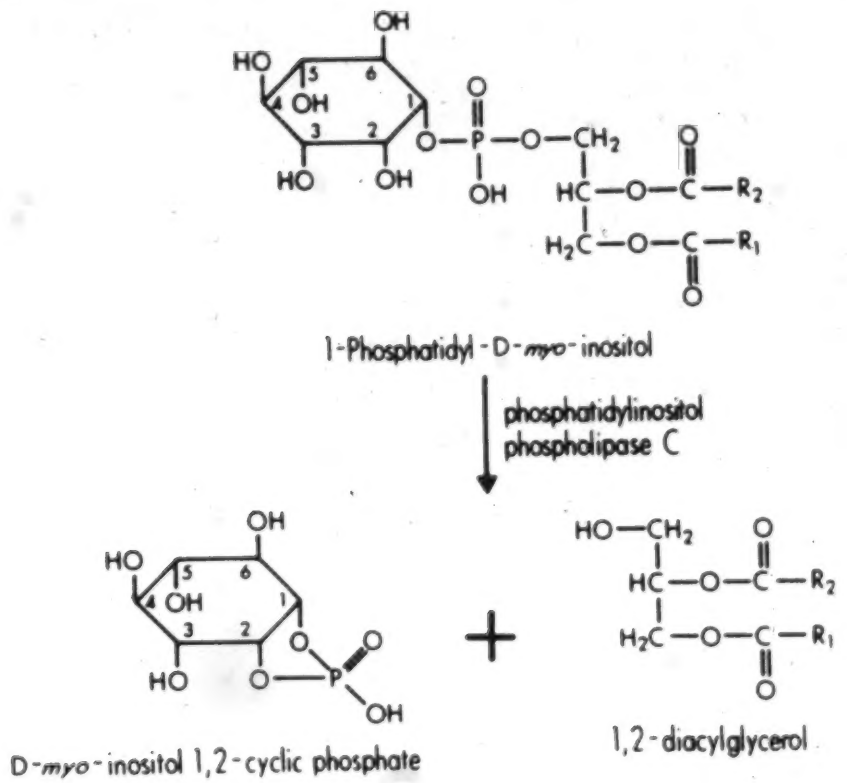


Figure 2. Reaction catalyzed by phosphatidylinositol phospholipase C.

TABLE 1: SOME PROPERTIES OF BACTERIAL C-TYPE PHOSPHATIDYLINOSITOL PHOSPHOLIPASES

Organism	Bacillus cereus	Bacillus thuringinsis	Clostridium novyi type A	Staphylococcus aureus
pI	5.4	5.4[a] or 4.9[a]	5.6[b] or 6.0[b]	N.D.[c]
Molecular mass	29,000[d]	23,000[a,e] or 22,000[a,e]	30,000[e]	20,000[e] or 33,000[f]
Inhibition by EDTA	No	No	No	No
Inhibition by 1,10-phenanthroline	No	No	No	No
Inhibition by divalent cations (Mg^{2+}, Ca^{2+}, Zn^{2+}, Mn^{2+})[g]	Yes	Yes	Yes	N.D.
Inhibition by monovalent cations (K^+, Na^+, NH_4^+)[g]	Yes	Yes	N.D.	Yes
References	4,7,8	9,10	11	12--15

[a] More than one strain was assessed.

[b] The pI values differ, but other properties were similar.

[c] N.D. (not determined).

[d] Gel filtration and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

[e] Gel filtration only.

[f] SDS-PAGE only.

[g] Not all cations were assessed with each PI-PLC.

It is possible that any substitution at positions 4 or 5 of the inositol ring of the PI could render it unsusceptable to S. aureus PI-PLC action. Thus, as far as protein anchorage is concerned, carbon positions 2, 3, and 6 on the inositol may be the likely sites for attachment of the glycan moiety of a PI-anchored protein. To support this postulate, it was found that PI--mannosides from Corynebacterium and Mycobacterium, which have mannosyl residues at positions 2 and 6 of the inositol ring (19,20), were hydrolyzed by this PI-PLC (18).

E. PHOSPHATIDYLINOSITOL--GLYCAN PHOSPHOLIPASE C ENDOGENOUS TO TRYPANOSOMES

In the early 1980's, it was shown that the simultaneous addition of Ca^{2+} and a calcium ionophore released about 80% of the variable surface glycoprotein (VSG) of Trypanosoma brucei (a parasitic protozoan) from the plasma membrane in 10 min, without rupture of the cell (21). The release of the VSG, a PI-anchored protein (22), could be inhibited by Zn^{2+} and was strongly temperature-dependent (23). When T. brucei cells were ruptured, variable surface glycoprotein in a membrane form (mfVSG) was transformed into a soluble variable surface glycoprotein (sVSG) (24). The mfVSG was shown to behave as an integral membrane protein because it was amphiphilic while the sVSG was not. Due to differing reactivities against an antibody directed at the C-terminal

region of VSG, it was concluded that the mfVSG and sVSG differ at the C-terminal domain (24). Together, these results suggested that the VSG of T. brucei could be released from the membrane by some endogenous enzyme that acted at or near the C-terminus of the VSG. When trypanosomes were labelled with [^{3}H] myristic acid, it was shown that the release of the VSG from the membrane occurred at the same time as the loss of myristic acid from this glycoprotein (25), implicating the possible role of a phospholipid(s) in the membrane anchorage of VSG. Later, it was shown that sn-1,2-dimyristylglycerol, a diacylglycerol, was released when mfVSG was converted to sVSG, showing that the endogenous, membrane-associated enzymatic activity responsible for this conversion was most likely a phospholipase C (26). The endogenous phospholipase responsible for this conversion was purified by three different groups (27--29). It was a membrane-bound enzyme, which on SDS-PAGE had a M_r of 37,000 to 39,800 (27--29). It accounted for approximately 0.1% of the total cellular protein (28) and based on hydrodynamic parameters, appeared to be monomeric in its native state (29). This enzyme was found to be stimulated by thiol-reducing agents or chelating agents and did not require Ca^{2+} for its activity (28,29). In general, the rate at which PI was cleaved by this phospholipase was much less than the rate at which mfVSG was cleaved (27--29), implying that the enzyme had a PI--glycan specificity. The term "glycan" refers to the structure, composed predom-

inantly of sugars, that acts as a chemical "bridge" between the PI membrane anchor and the protein to which it is attached (see Section H of this chapter). This trypanosomal phospholipase is unlike the bacterial PI-PLCs (see Section D of this chapter), which do not require the presence of a glycan on the PI in order to be able to hydrolyze it (7,8,10). Also, phosphatidylglycerol, phosphatidylethanolamine, and phosphatidylcholine could not serve as substrates for the trypanosomal enzyme (27). Further studies have shown that this phospholipase activity was predominantly associated with the flagella and may be localized in the membrane of the flagellar pocket of the trypanosomes (30).

F. THE INSULIN-STIMULATED PHOSPHATIDYLINOSITOL--GLYCAN PHOSPHOLIPASE C

Many of the metabolic effects of insulin could be due to the generation from the plasma membrane of a substance or group of substances which regulate certain enzymes (31). In murine myocyte cells and a bovine liver particulate fraction, it was found that after insulin bound to its receptor, one or two complex carbohydrates were released from the membrane (32--34). These complex carbohydrates contained inositol, glucosamine, phosphate (32,33), and other monosaccharides (34). The complex carbohydrates exhibited a net negative charge, even at pH 1.9, and an M_r of approximately 1,400 (33). Diacylglycerol was also produced upon release of the complex carbohydrates from the

membranes (32). An exogenously added bacterial PI-PLC also resulted in the formation of the same complex carbohydrates and diacylglycerol, and this led to the conclusion that an endogenous phospholipase C was causing the release of these carbohydrates (32,35). These complex carbohydrates could modulate the activity of insulin-sensitive enzymes such as adenosine 3',5'-monophosphate phosphodiesterase, adenylate cyclase, and pyruvate dehydrogenase (32--35). Therefore, insulin, on binding to its receptor, stimulates an endogenous phospholipase C.

Later, a PI--glycan phospholipase C from rat liver plasma membranes, which may be responsible for the insulin-induced release of the complex carbohydrate enzyme modulators, was partially purified (36). The enzyme had a subunit M_r of 52,000 and was a monomer in its active form. It was Ca^{2+}-independent, thiol-activated, and was found to be specific for glycosylated PI; no hydrolysis of PI, PI-4,5-bisphosphate, or phosphatidylcholine was observed.

G. PROTEINS ANCHORED TO THE MEMBRANE BY PHOSPHATIDYLINOSITOL

Many proteins have now been shown to be anchored to the plasma membrane by a covalent linkage to PI. These are listed in Table 2. The proteins involved have been found in a wide variety of eukaryotic organisms, from protozoan parasites to man. As of yet, PI-anchored proteins have not been found in prokaryotes or in higher plants. Not only are these proteins found in a diverse number of organisms, but

TABLE 2: PROTEINS THOUGHT TO BE ANCHORED TO MEMBRANES VIA COVALENT LINKAGE TO PHOSPHATIDYLINOSITOL

	Reference
A. Proteins possessing enzymatic activity.	
1. Alkaline phosphatase	4,14
2. 5'-Nucleotidase	37,38
3. Acetylcholinesterase	39,40
4. Alkaline phosphodiesterase I	41,42
5. Trehalase	43
6. *Leishmania* surface protease	44,45
7. Renal dipeptidase	46
8. Arylacylamidase	47
B. Proteins not known to possess an enzymatic activity.	
1. Thy-1 antigen	48--50
2. Trypanosome variable surface glycoprotein	22,26
3. Neural cell adhesion molecule	51,52
4. RT-6.2 antigen	53
5. T-cell activating protein	54
6. Qa antigen	55
7. ThB antigen	55
8. Heparin sulfate proteoglycan	56
9. Ly-6 A, C, and E antigens	57
10. Rat hepatoma major glycoprotein	58
11. Decay accelerating factor	59,60
12. Lymphocyte function-associated antigen	61
13. Carcinoembryonic antigen	62
C. Protein suspected to be an infectious agent.	
1. Scrapie prion protein	63

the proteins themselves are diverse in their biochemical characteristics. Some, such as alkaline phosphatase and acetylcholinesterase, possess enzymatic activity while others, such as Thy-1 and RT-6, possess no known enzymatic activity. Even though not much is known about the in vivo function of many of these proteins, they probably are very diverse in function.

H. STRUCTURE OF THE "BRIDGE REGION" OF PHOSPHATIDYLINOSITOL-ANCHORED PROTEINS

The term "bridge region" refers to the region composed primarily of sugar residues (i.e. glycan) between the PI anchor and the carboxy terminus of the protein. Partial chemical characterization of the "bridge region" of a number of PI-anchored proteins has been done, and a general model of the "bridge region" can be inferred by putting together the data derived so far (Figure 3).

Rat brain Thy-1 was the first PI-anchored protein to be shown to have glucosamine present in its bridge (64). The position of the glucosamine relative to the PI anchor was first ascertained in 1985 when it was shown that in trypanosome VSG, there was a single non-N-acetylated glucosamine residue that was in a glycosidic linkage to myo-inositol monophosphate (22). Cleavage of the glycosidic bond at the 1-position of the glucosamine of human erythrocyte acetylcholinesterase and human placental alkaline

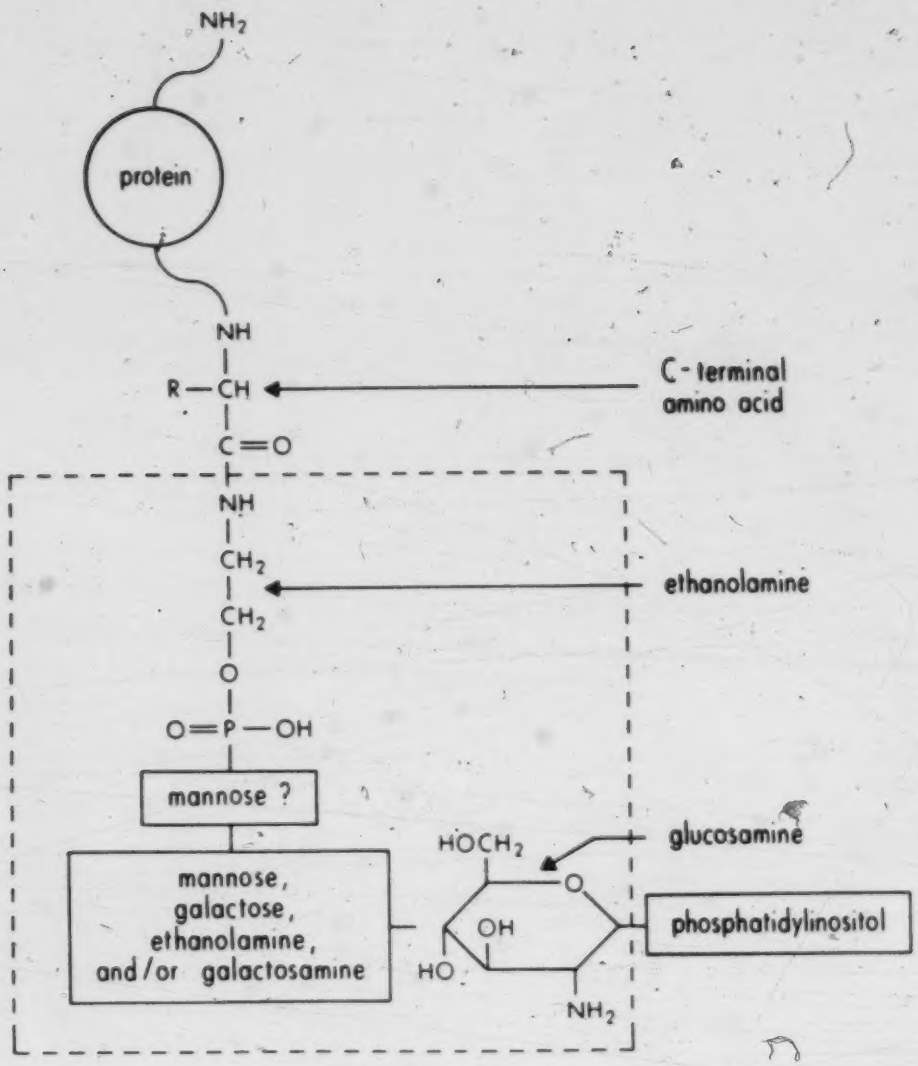


Figure 3. Proposed model of the "bridge region" of PI-anchored proteins.

phosphatase by nitrous acid released fatty acids and inositol from the protein, confirming that glucosamine is bound directly to the PI (65,66). Also, human erythrocyte decay accelerating factor (DAF) and acetylcholinesterase have been shown to have, in their C-terminal membrane anchors, glucosamine in a 1:1 ratio with each polypeptide (60). Lastly, acetylcholinesterase from Torpedo californica electric organ and alkaline phosphatase from bovine intestine were shown to have a sugar residue (not definitively identified, but probably glucosamine), bearing a free amino group, that is attached through a glycosidic linkage to PI (67). All of the above information leaves little doubt that glucosamine is a constituent of the "bridge region" of most, if not all, PI-anchored proteins.

Human erythrocyte DAF has ethanolamine attached to its C-terminus as part of the "bridge region" of the membrane anchor (60). Also, work with Thy-1 showed that [^{3}H]-ethanolamine was incorporated almost exclusively into the hydrophobic, membrane-anchoring fragment of this protein but this compound was not incorporated into the Thy-1 formed by a cell line in which this protein is secreted as a hydrophilic species (68). This also suggested an association of ethanolamine with the membrane-anchoring region. Finally, in T. brucei VSG and human erythrocyte acetylcholinesterase, the ethanolamine has been shown to be linked via an amide bond to the α-carboxy group of the C-terminal amino acid

(for VSG, either an aspartic acid or a serine; for the acetylcholinesterase, a glycine) (69,70).

The structure of the remainder of the "bridge region" is less certain. Mannose is probably linked to the ethanolamine through a phosphate; partial acid hydrolysis of the C-terminal glycopeptide of trypanosome VSG released ethanolamine phosphate and mannose-6-phosphate (71). Between this mannose and the glucosamine that is linked to the PI is the glycan region. Mannose, galactose, ethanolamine, and/or galactosamine are the likely constituents of this region. Evidence for this is as follows: the carboxy-terminus of T. brucei VSG has been shown to have a mannose/C-terminus molar ratio of approximately 2:1 and a galactose/C-terminus molar ratio of approximately 4:1 (22). Also, the carboxy terminii of rodent brain and thymocytic Thy-1 shows a mannose/C-terminus molar ratio of 2:1, an ethanolamine/C-terminus molar ratio of 2:1, and the presence of galactosamine in the C-terminus (49).

I. BUTANOL-STIMULATED PHOSPHOLIPASES

Alkaline phosphatase from rat liver plasma membranes, when extracted with 1-butanol at a pH >7.0, was not electrophoretically identical to the serum form derived from the liver (72,73). However, hepatic alkaline phosphatase released from the plasma membranes by a bacterial PI-PLC was found to be electrophoretically identical with the serum

form (73). When the butanol extractions were done at pH values <7.0, the extracted alkaline phosphatase was electrophoretically identical to the serum form, the activity causing this conversion being detected in the plasma and lysosomal membranes of the rat liver (74). The alkaline phosphatases from certain rat tissues (liver, kidney, and intestine) that were extracted with 1-butanol at pH 5.5 or 8.5 (serum and membranous forms, respectively), were purified. No significant differences in amino acid composition was found between these two forms, suggesting possible involvement in the membranous forms of a hydrophobic component other than its polypeptide domain (75). The general finding that the soluble form of alkaline phosphatase would be produced when the butanol extracts were done at a slightly acidic pH hinted that there may be an endogenous enzyme activity responsible for this process, since most enzymatic reactions are pH dependent.

Butanol extracts at two different pH values (5.4 and 8.3) were also carried out on a particulate fraction from human placenta (76). The alkaline phosphatase extracted at the lower pH was found to have an M_r of approximately 170,000 and a relatively low hydrophobicity, the same result as that obtained with Staphylococcus aureus PI-PLC. However, butanol extraction at pH 8.3 solubilized an alkaline phosphatase that aggregated with an M_r of approximately 600,000, and possessed a relatively high hydrophobicity. The formation of the low M_r alkaline phosphatase during the butanol extrac-

tion was found to be a Ca^{2+} - and thiol-dependent process, and unaffected by protease inhibitors or heat. A butanol-stimulated activity which removed [^{3}H] myristic acid from the VSG of T. brucei was detectable in the human placental particulate fraction. Since this activity was active below pH 7.0, Ca^{2+} - and thiol-dependent and relatively heat-stable, it may be the same as that responsible for the production of the low M_r alkaline phosphatase. The [^{3}H] mfVSG-cleaving activity was probably a phospholipase D since the only [^{3}H] product identified was phosphatidic acid (refer to Figure 1). These data suggested that the production of low M_r alkaline phosphatase during butanol extraction was due to an endogenous phospholipase.

J. POSSIBLE ROLES FOR A PHOSPHATIDYLINOSITOL MEMBRANE ANCHOR

This is the most intriguing aspect of the subject of PI anchorage for proteins. Presumably, this linkage allows the protein to better perform its physiological function than if it had been anchored to the membrane in a more conventional manner (i.e. via a hydrophobic polypeptide). A logical way to begin to investigate the question of "Why?" is to ask "What kind of general properties can be conferred on any protein bound to a PI anchor?". Some of these are as follows:

1) Increased lateral mobility in the plasma membrane.

Since the PI anchor is a lipid, for energetic and

steric reasons, it should be able to diffuse laterally amongst the lipids making up the membrane at a much faster rate than a conventional hydrophobic peptide anchor; the peptide anchor is more bulky than a lipid and doesn't mesh as well with adjacent lipids as a PI anchor would. This property is almost a general certainty since work has been done on three different PI-anchored proteins, and the lateral mobility of all three have been shown to be substantially faster than that of other membrane proteins; these proteins are Thy-1, DAF, and alkaline phosphatase. They were shown to have diffusion coefficients that ranged from $0.6--3.9x10^{-9}$ cm^2/s (77--79). These lateral diffusion rates are at the low end of the range of diffusion coefficients for lipid probes (range approximately $0.2--2.0x10^{-8}$ cm^2/s) and are an order of magnitude higher than the coefficients for "typical" membrane proteins (range approximately $0.5--6.0x10^{-10}$ cm^2/s) (77--80). With DAF, it has been shown that <100 molecules need to be present on a red cell in order to inhibit complement activation on the surface of the cell (81). The accomplishment of this function with such a small number of DAF molecules could be attributed to an above average lateral mobility since the higher the lateral mobility of the DAF molecule, the greater the probability that it will be able to come in contact with, and neutralize, any complement that comes near the plasma

membrane of the red cell.

2) Facilitated release of attached proteins.

Proteins bound to the plasma membrane by a PI anchor can be selectively released from the membrane by certain phospholipases. Differing degrees of metabolic control could be achieved by the use of phospholipases of varying selectivity. The lowest degree of control would be the use of phopholipases that are similar in nature to the bacterial PI-PLCs. These enzymes are relatively non-selective and not only will release PI-bound proteins, but will also cleave off the free inositol headgroups of PI itself (see Section D of this chapter). So far, phospholipases that are this non-selective and that can release PI-bound proteins have not been isolated from eukaryotes. A higher degree of metabolic control could be conferred by utilizing phospholipases that are similar to the insulin-stimulated PI--glycan PLC or the trypanosome PI--glycan PLC in specificity. These enzymes recognize PI--glycans and not just the PI moiety itself (see Sections E and F of this chapter). The highest degree of metabolic control, although totally speculative at this time, would be through the use of phospholipases that recognize only specific proteins that are bound to PI anchors, thereby releasing only these proteins from the membrane while leaving other PI-anchored proteins attached to the same membrane.

An example of a system in which particular physio-

logical roles have been attributed to the release of a PI-anchored protein is with the trypanosome VSG. These roles are: (a) antigenic variation in order to evade the immune system in the trypanosome's mammalian host, and: (b) shedding of the VSG once the trypanosome has been ingested into the midgut of the tsetse fly (82,83). However, definitive evidence showing that the PI--glycan-PLC characterized in the trypanosome (see Section E of this chapter) is responsible for these in vivo phenomena has not been established, although there is a good probability that this enzyme does play a role in at least one of these phenomena.

3) Recognition and binding of PI-anchored proteins to cell-surface receptors.

The rationale behind this concept is that once a PI-bound protein has been released from the membrane by a PI-PLC or a PI--glycan-PLC, an unusual glycan should be exposed at the C-terminus of the soluble protein. This glycan could serve as a site for specific recognition and binding by a surface receptor on the same or another cell. Evidence for this type of process has recently been obtained from studying the heparin sulfate proteoglycan (HSPG) from rat hepatocytes (56). This protein is initially bound to the plasma membrane via PI and is subsequently cleaved from the cell surface by an insulin-activated PI-PLC. The solubilized HSPG can then attach to a

cell surface receptor that recognizes the myo-inositol-phosphate moiety that is present on the HSPG and upon attachment, is internalized by the cell. Inside the cell, a portion of the HSPG is converted to free heparin sulfate chains which eventually appear in the nucleus.

As further work is done on the functions of PI-anchored proteins, more details will be revealed about the three aforementioned properties that can be conferred on these proteins, and about how these properties may serve a physiological function. Also, future work may uncover as yet unknown properties that could be conferred to PI-anchored proteins. The functions that can be given to a protein by a PI anchor is one of the least-studied aspects of the PI-anchored proteins and as a result, is one of the most promising and interesting areas in which future work is to be done.

K. ALKALINE PHOSPHATASE: AN OVERVIEW

Alkaline phosphatase (orthophosphoric monoester phosphohydrolase [alkaline optimum] EC 3.1.3.1) is ubiquitous in living organisms, being present in almost every category of organisms with the exception of the higher plants (84). This enzyme can catalyze the transfer of phosphoryl groups from phosphomonoesters to either water (phosphohydrolysis) or to other hydroxyl-containing acceptors (transphosphorylation) (85), and is relatively non-specific in its choice of sub-

strates (86,87). Some of the phosphomonoesters that can be used as substrates during phosphohydrolysis are glycerophosphates, ethyl phosphate, hexosephosphates, and phenyl phosphate (88,89). During transphosphorylation, glucose, glycerol, and propanediol have been shown to accept phosphoryl residues from a wide variety of donors (90). Proteins can also be dephosphorylated by alkaline phosphatase, one of the first examples of this being the dephosphorylation of lysine-rich histones by E. coli alkaline phosphatase (91,92).

In mammals, alkaline phosphatase is not catalytically active in a monomeric form (86). Zn^{2+} is essential for its activity (93) while Mg^{2+} is an activator (94). Alkaline phosphatase can also exist as a tetramer, as was first shown for the pig kidney enzyme (95). Subsequently, tetrameric enzyme has been shown in choriocarcinoma cells (96), human first trimester placenta (97), chicken epiphyseal cartiledge (98), chick kidney (99), and human liver (100). In humans, alkaline phosphatase is a membrane-bound enzyme that is coded for by at least four genes (101,102). Functionally, alkaline phosphatase has been implicated in bone mineralization (103,104) and protein dephosphorylation (105--108). Also, phosphate transport has been proposed as another function (109,110), although there has been fairly convincing evidence to dispute this (111,112).

CHAPTER TWO

RELEASE OF ENZYMES FROM HUMAN LIVER PLASMA MEMBRANES BY A BACTERIAL PHOSPHATIDYLINOSITOL PHOSPHOLIPASE C

A. INTRODUCTION

In the present study, I sought to determine some of enzymes which were or were not released from human liver plasma membranes by a purified PI-PLC obtained from Bacillus cereus.

B. MATERIALS AND METHODS

1. Chemicals

Phospholipase C (Bacillus cereus) type III, p-nitrophenylthymidine 5'-monophosphate, p-nitrophenylphosphate, phenylmethylsulfonyl fluoride (PMSF), leupeptin, bestatin, pepstatin A, and naphthol AS-MX phosphate were purchased from Sigma (St Louis, MO), ethylaminoethanol from Aldrich (Milwaukee, WI), and the Enzyline 5'-nucleotidase assay kit from bioMérieux (France). All other chemicals were reagent grade (Fisher Scientific, Fairlawn, NJ).

2. Plasma Membrane and Enyme Preparation, and Enzyme Assays

Plasma membranes were isolated as previously described by this laboratory (100) and PI-PLC was purified from a commercial preparation of nonspecific phospholipase C (8). Alkaline phosphatase was assayed in 1 ml of medium containing 10 mmol/l p-nitrophenylphosphate, 1.0 mol/l ethyl-

aminoethanol, pH 10.3, at 30°; the increase in absorbance at 404 nm was monitored with a spectrophotometer, (Varian, model 2200). Enzyme activity (U/l) was expressed as μmol of p-nitrophenol released/min/l. Alkaline phosphodiesterase I activity was assayed in 1 ml of medium containing 2 mmol/l p-nitrophenylthymidine 5'-monophosphate , 100 mmol/l Tris, 1 mmol/l Mg^{2+} , pH 8.9, at 30°; the increase in absorbance at 404 nm was measured with a spectrophotometer, (Varian, model 2200). The activity of 5'-nucleotidase was assayed using the Enzyline 5'-nucleotidase kit obtained from bioMérieux.

3. Time Courses of Enzyme Release by PI-PLC

Aliquots of isolated plasma membranes (alkaline phosphatase activity = 600 U/l) suspended in a buffer containing 50 mmol/l Tris, 10% (v/v) glycerol, pH 7.6, were incubated with equal volumes of PI-PLC in the same buffer at 30°. Final PI-PLC concentrations were from 0 (control) to 5.0 μg/ml and incubation times were from 15 to 75 min. At each incubation time, an aliquot of each sample was centrifuged at 95,000xg for 8 min; enzyme activity recovered from the supernatant fluid was expressed as a percentage of the activity in the uncentrifuged control.

C. RESULTS AND DISCUSSION

The amount of alkaline phosphatase released from the liver plasma membranes was proportional to the PI-PLC

concentration and the duration of treatment at a fixed PI-PLC concentration (Figure 4). Inhibitors of proteolytic enzymes (0.02% (w/v) PMSF, 1 μmol/l leupeptin, 0.23 mmol/l bestatin, and 0.10 mmol/l pepstatin A) did not inhibit the PI-PLC mediated release of the enzyme. The enzyme 5'-nucleotidase was also released in a manner very similar to that of alkaline phosphatase (Figure 5). Alkaline phosphodiesterase was not released from the membranes even by 1 h of treatment with 30 μg/ml of PI-PLC, even though this enzyme has been shown to be released by PI-PLC from the plasma membranes in other mammalian tissues (41,42).

By analogy with the release of membrane-bound alkaline phosphatase and 5'-nucleotidase from various non-human mammalian tissues by bacterial PI-PLCs (4,14,38), it is very likely that the anchorage of alkaline phosphatase and 5'-nucleotidase to the plasma membrane in human liver is via a covalent bond with PI (11,14). It is highly unlikely that proteolytic activity was a cause of this release, in view of the lack of inhibition of this release by protease inhibitors. Though the alkaline phosphodiesterase I was not released from human liver plasma membranes by PI-PLC, the possibility of a covalent linkage to PI for this enzyme cannot be ruled out since the bond cleaved by the PI-PLC may be unaccessable.

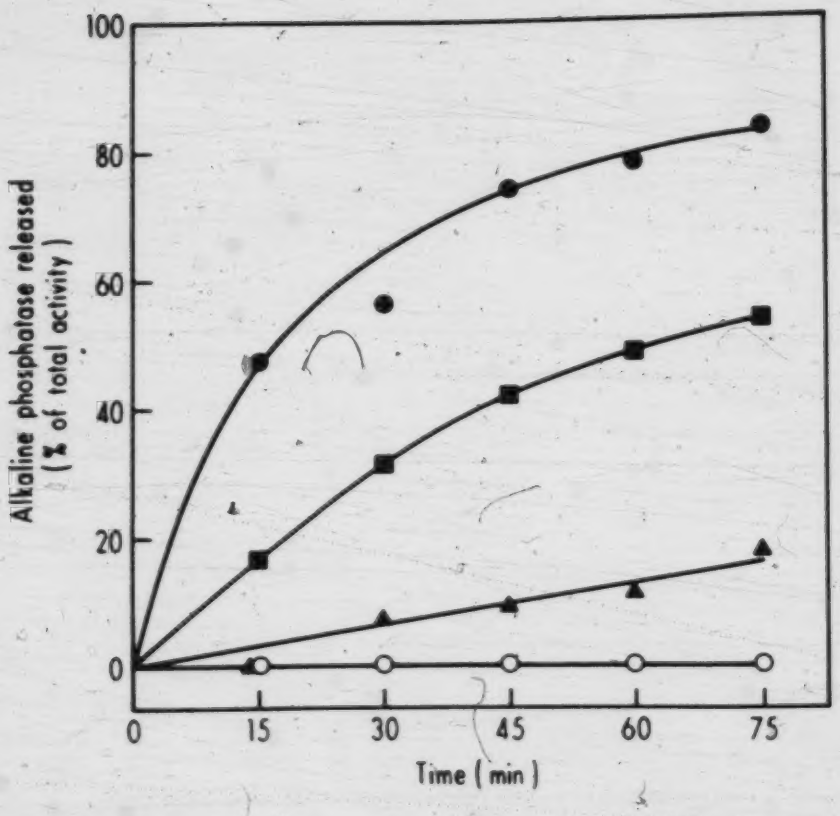


Figure 4. Solubilization of membrane-bound alkaline phosphatase by PI-PLC applied in the following concentrations: ○, 0 μg/ml; ▲, 0.2 μg/ml; ■, 1.0 μg/ml; and ●, 5.0 μg/ml.

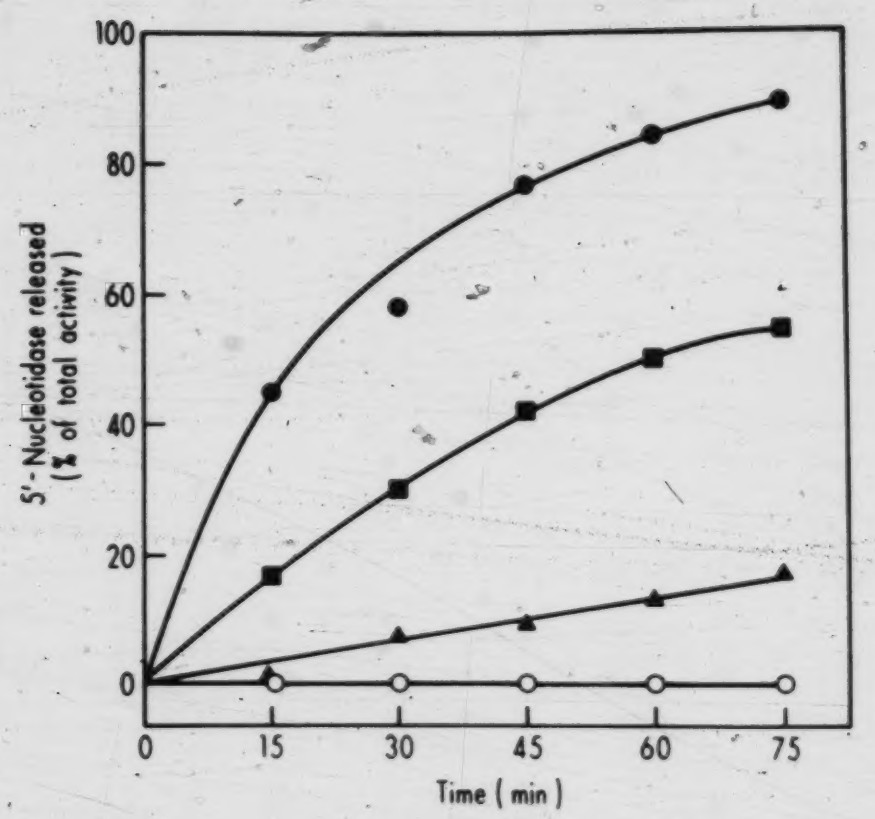


Figure 5. Solubilization of membrane-bound 5'-nucleotidase by PI-PLC applied in the following concentrations: ○, 0 μg/ml; ▲, 0.2 μg/ml; ■, 1.0 μg/ml; and ●, 5.0 μg/ml.

CHAPTER THREE

THE SOLUBILIZATION OF TETRAMERIC ALKALINE PHOSPHATASE FROM HUMAN LIVER AND ITS CONVERSION INTO VARIOUS FORMS BY PHOSPHATIDYLINOSITOL PHOSPHOLIPASE C OR PROTEOLYSIS

A. INTRODUCTION

Alkaline phosphatase (orthophosphoric-monoester phosphohydrolase [alkaline optimum], EC 3.1.3.1) purified from human liver is a dimeric glycoprotein of M_r 135,000--186,000 (113,114). As the enzyme from this source can be released from the plasma membrane by a bacterial PI-PLC, it is most likely anchored to this membrane via a covalent attachment to PI (87,115). Membrane-bound alkaline phosphatases from other mammalian tissues can also be released by various bacterial PI-PLCs (9,11,14). This type of anchorage may also apply to other eukaryotic membrane-bound proteins (11,41,43,58,115--117).

Human liver alkaline phosphatase, when purified from a butanol extract of a liver homogenate carried out at pH 6.6, is dimeric in its quaternary structure (118). However, this enzyme may be tetrameric in its native, membrane-bound state (100) and in the present study, we have shown that when the enzyme is purified from a butanol extract of a liver homogenate carried out at pH 8.5, it is predominantly in a tetrameric state. We have used PI-PLC and proteolytic enzymes as probes to study the quaternary structure of and

the membrane anchorage of human liver alkaline phosphatase, and employed these and others' data to construct a possible model of the enzyme in human liver plasma membranes.

B. MATERIALS AND METHODS

1. Chemicals

Phospholipase C (*Bacillus cereus*) type III, ficin, bromelain, subtilisin Carlsberg, Nonidet P-40 (NP40), Triton X-100, p-nitrophenylphosphate, naphthol AS-MX phosphate, dipalmitoyl phosphatidylcholine, 2-amino-2-methyl-1-propanol, and PMSF were purchased from Sigma (St Louis, MO), ethylaminoethanol from Aldrich (Milwaukee, WI), M_r markers for gradient gel electrophoresis and octyl-Sepharose CL-4B from Pharmacia (Uppsala, Sweden), acrylamide, N,N'-methylenebisacrylamide, sodium dodecyl sulfate (SDS), and SDS molecular weight protein markers were from Bio-Rad (Richmond, CA), and 2.5--27% polyacrylamide gradient gels in rods from Isolab (Akron, OH). All other chemicals were reagent grade (Fisher Scientific, Fairlawn, NJ).

2. Plasma Membrane and Enzyme Preparation, and Enzyme Assay

The protocols for the preparation of the human liver plasma membranes, the purification of the PI-PLC, and of the kinetic assay for the alkaline phosphatase were described in Chapter Two, Section B. Dimeric alkaline phosphatase was purified from a butanol extract of a liver homogenate carried out at pH 6.6 as described previously (119).

3. Alkaline Phosphatase Release from Plasma Membranes with Proteases

Aliquots of isolated plasma membranes (alkaline phosphatase activity approximately 600 U/l) were suspended in a buffer containing 50 mmol/l Tris, 10% (v/v) glycerol, pH 7.6 (hereafter known as Tris buffer). Test samples were incubated with equal volumes of bromelain or ficin (final concentration, 5 mg/ml) in the same buffer at 30°. No reducing agents were added to the protease preparations. The control sample contained no protease. Incubation times were from 0 to 30 h. At specified times, an aliquot of each sample was centrifuged at room temperature for 15 min at 90,000xg; enzyme activity recovered from the supernatant fluid was expressed as a percentage of the activity in the corresponding uncentrifuged sample.

4. Gradient and SDS Polyacrylamide Gel Electrophoresis

The native M_r of alkaline phosphatase in each sample (100 µl, alkaline phosphatase activity approximately 250 U/l) was determined by electrophoresis in gradient gels of polyacrylamide (2.5--27%) for 18h at 150 V in a buffer containing 0.09 mol/l Tris, 0.08 mol/l boric acid, pH 8.4 (114). No detergents were present in the electrophoresis buffer. The gels were stained using as a substrate medium, 3.0 mg/ml naphthol AS-MX phosphate, 1.0 mol/l ethylamino-ethanol, 1.5 mmol/l Mg^{2+}, pH 10.3. Fluorescing band(s) indicating alkaline phosphatase activity were photographed

under UV light (114), and the M_r markers were stained with Coomassie blue G-250 (120).

The subunit M_r of alkaline phosphatase was determined by polyacrylamide gel electrophoresis in the presence of SDS (121). The support consisted of a stacking gel (3% (w/v) acrylamide) and a resolving gel (10% (w/v) acrylamide). Each sample of enzyme (3 U of activity) was added to an equal amount of a buffer containing 2% (w/v) SDS, 5% (v/v) β-mercaptoethanol, 10% (v/v) glycerol in 62.5 mmol/l Tris, pH 6.8. The samples were then heated in boiling water for 3 min before tracking dye (0.1% (w/v) bromphenol blue, final concentration) was added. The samples (50-75 µl) were applied to the gel, and electrophoresis was carried out at a constant current of 30 mA in a Bio-Rad Protean II apparatus. Proteins were stained with Coomassie Blue R-250 (114).

5. Treatment of Detergent-solubilized Tetrameric Alkaline Phosphatase with PI-PLC

NP40 was added to purified plasma membranes (alkaline phosphatase activity approximately 600 U/l) suspended in the Tris buffer to give 1% (v/v) NP40. Samples were mixed at 4° for 1h and then centrifuged for 15 min at 95,000xg. The supernatant fluid (containing the detergent-solubilized enzyme) was divided into three aliquots: to one aliquot (control) was added an equal amount of the Tris buffer containing no PI-PLC; to the other two was added an equal volume of PI-PLC (in the Tris buffer) to give final PI-PLC concentrations of 5.0 and 57.4 µg/ml, respectively. The

samples were incubated at 30° for 1h. NP40 was then added to all three incubation mixtures, to a final concentration of 6% (v/v), before application to gradient gels.

6. Treatment of Detergent-solubilized Tetrameric Alkaline Phosphatase with Subtilisin Carlsberg

NP40 was added to purified plasma membranes (alkaline phosphatase activity approximately 600 U/l) suspended in the Tris buffer, to give 1% (v/v) NP40. This was mixed at 4° for 1 h, then centrifuged at room temperature for 15 min at 90,000xg. The supernatant fluid was divided into three aliquots: to one (control) was added an equal amount of the Tris buffer; to each of the other two aliquots was added an equal volume of subtilisin Carlsberg in the Tris buffer, to give final subtilisin concentrations of 0.5 mg and 5.0 mg/ml, respectively. The samples were incubated at 30° for 3 h. NP40 was then added to all three incubation mixtures, to a final concentration of 6% (v/v), before application to gradient gels.

7. PI-PLC Treatment of a Butanol Extract of NP40-Solubilized Alkaline Phosphatase

NP40 solubilization was accomplished as described above. Following centrifugation, NP40 was added to the supernatant fluid to a final concentration of 6% (v/v). To an aliquot of this supernatant was added an equal volume of cold (-20°) 1-butanol. The mixture was put on a wrist-action shaker for 1 h at 4°, and centrifuged at 10,000xg for 10 min at room temperature. The aqueous layer was recovered and PI-PLC

added to a final concentration of 50.1 μg/ml and the sample incubated at 30° for 3 h before application to gradient gels.

8. Purification of Tetrameric Alkaline Phosphatase

All procedures were carried out at 4° unless stated otherwise. Human liver (100 g) obtained at autopsy and stored as described above was thawed and homogenized in a Waring Blender in 100 ml of 50 mmol/l Tris, 1 mmol/l Mg^{2+}, 0.1 mmol/l Zn^{2+}, 0.1% (w/v) PMSF, 100 μmol/l leupeptin, 20 μmol/l pepstatin, pH 8.5, for 5 min at low speed followed by 5 min at high speed. Organic solvent, 1-butanol, (200 ml) cooled to -20° was added and the mixture stirred for 1 h and then centrifuged at 9000xg for 30 min. The aqueous layer was recovered and recentrifuged as above to sediment any remaining particulate matter. The aqueous layer was then applied to a monoclonal antibody column (specific for liver-type alkaline phosphatase) of 4 ml and washed with several column volumes of 50 mmol/l Tris, 1 mmol/l Mg^{2+}, 0.1 mmol/l Zn^{2+}, 0.1% (v/v) Triton X-100, pH 8.5, at 0.5 ml/min. The enzyme was eluted with 0.8 mol/l 2-amino-2-methyl-1-propanol, pH 10.3, pooled, and dialyzed extensively against 50 mmol/l Tris, 1 mmol/l Mg^{2+}, 0.1 mmol/l Zn^{2+}, pH 8.5. To remove the detergent an equal volume of 1-butanol at -20° was added and a butanol extraction was carried out as before. The enzyme was concentrated and small amounts of dimer removed by adsorption to octyl-Sepharose. The enzyme was added at 20° to 3.5 ml of packed beads suspended in 50 mmol/l Tris, 1 mmol/l Mg^{2+}, 0.1 mmol/l Zn^{2+}, pH 8.5. The beads were gently mixed

for 15 min at 20° and centrifuged at 12,000xg for 20 min, recovered, washed in the above buffer and the enzyme eluted in this buffer plus 10% (v/v) Triton X-100.

9. Hydrophobic Chromatography

Enzyme released by PI-PLC was prepared by incubating liver plasma membranes with 6 μg/ml of PI-PLC at 30° for 2.5 h. The ficin-released form of the enzyme was prepared by incubating liver plasma membranes with 5 mg/ml of ficin at 30° for 8 h. The membranes were removed after both treatments by centrifugation at 90,000xg for 15 min at room temperature. The butanol-extracted form of the enzyme was prepared by mixing equal volumes of liver plasma membranes with 1-butanol (precooled to -20°) and shaking this mixture for 1 h at 4° on a wrist-action shaker. The butanol-membrane mixture was then centrifuged at 10,000xg for 10 min at room temperature and the aqueous layer containing the alkaline phosphatase activity recovered. The detergent-solubilized enzyme was prepared by mixing liver plasma membranes with Triton X-100 (1% (v/v), final concentration) for 1 h at 4° and spinning at 90,000xg for 15 min at room temperature to recover the supernatant. The butanol-extracted and Triton-solubilized forms of the enzyme were incubated for 2.5 h at 30° with (or without) 6 μg/ml PI-PLC. The purified dimer was obtained by a previously described method (119).

Alkaline phosphatase (0.15 U for the ficin-released and Triton-solubilized forms, and 0.25 U for the other forms, in approximately 400 μl) was applied to a 1 ml column of

octyl-Sepharose CL-4B at room temperature and the flow stopped for 5 min. The column was washed with four column volumes (collected in fractions of approximately 0.4 ml) of Tris buffer and then 500 µl of a 10% (v/v) solution of Triton X-100 in Tris buffer was applied. The column was allowed to sit for 15 min before another four column-volumes were collected (approximately 0.4 ml per fraction). Each fraction was assayed for alkaline phosphatase activity as described above.

10. Liposome Preparation and Alkaline Phosphatase Incorporation

Liposomes were prepared by dissolving 6 mg of dipalmitoyl phosphatidylcholine in 1 ml of chloroform and drying under nitrogen. A volume of 1 ml of the Tris buffer was then added and the mixture was sonicated (under nitrogen) on a bath sonicator for 15 min. Forms of alkaline phosphatase as described above were added to an equal volume of liposomes and mixed gently for 18 h at room temperature. The liposomes were sedimented by centrifugation at 90,000x*g* for 15 min at room temperature, and the supernatant and resuspended liposomes were assayed for alkaline phosphatase activity.

C. RESULTS

Although both ficin and bromelain solubilized the liver alkaline phosphatase of purified plasma membranes, the release by ficin took less time (Figure 6). The released enzyme was dimeric, with a M_r -- approximately 214,000 --

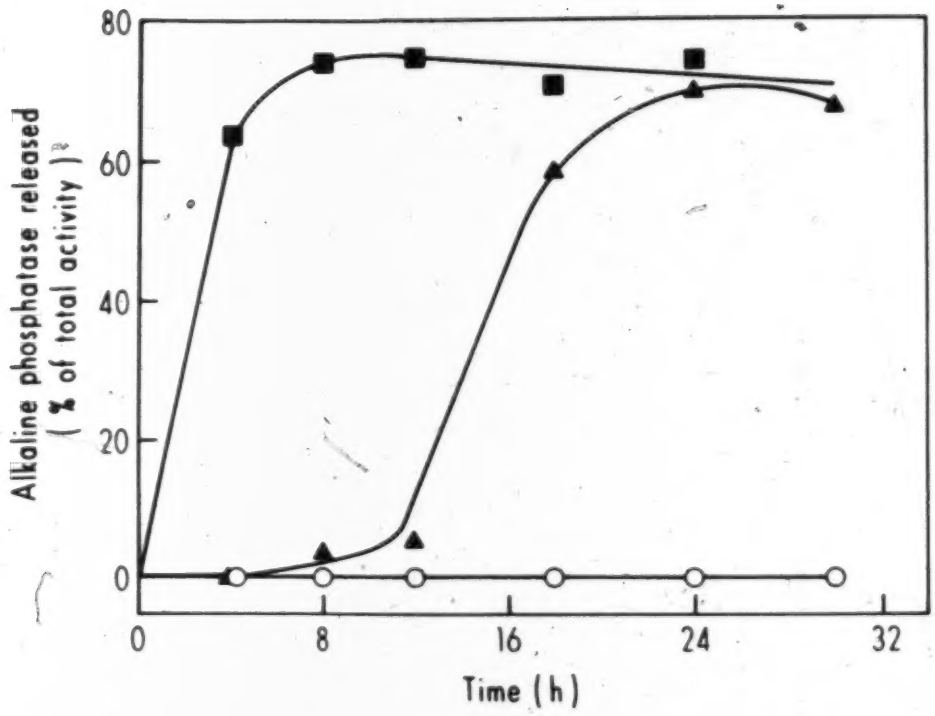


Figure 6. Solubilization of membrane-bound alkaline phosphatase by proteolytic enzymes: ○, control; ▲, bromelain, 5.0 mg/ml; and ■, ficin, 5.0 mg/ml.

the same as that for the enzyme purified from a butanol extract done at pH 6.6 (Plate 1).

PI-PLC converted detergent-solubilized tetrameric enzyme to dimers (Plate 2), to a degree proportionate to the PI-PLC concentration (compare lanes e and f). Proteolysis by subtilisin Carlsberg converted the detergent-solubilized tetrameric enzyme to dimers; this process, which was concentration-dependent, was inhibited by PMSF (0.02%, w/v), an inhibitor of serine proteases (122) (Plate 3). Ficin destroyed a significant amount of the activity of the detergent-solubilized alkaline phosphatase, with little or no conversion of tetramer to dimer, whereas bromelain did not inactivate the alkaline phosphatase activity but also was less competent than subtilisin Carlsberg in converting tetramer to dimer (results not shown).

Butanol extraction of the NP40-solubilized alkaline phosphatase produced a water-soluble enzyme, that on electrophoresis had a high $\underline{M}_r$ and that PI-PLC converted to dimers (Plate 4). The same is true of the enzyme directly extracted from membranes by butanol and subsequently treated with PI-PLC (results not shown). The tetrameric form of the enzyme solubilized by non-ionic detergent and purified by immunoaffinity adsorption was shown to have a subunit $\underline{M}_r$ of 83,000 (Plate 5). Dimeric enzyme formed from PI-PLC treatment of purified tetramer, and the enzyme purified from a butanol extract of whole liver carried out at pH 6.6 were also shown to have a subunit $\underline{M}_r$ of 83,000 (Plate 5). Alka-

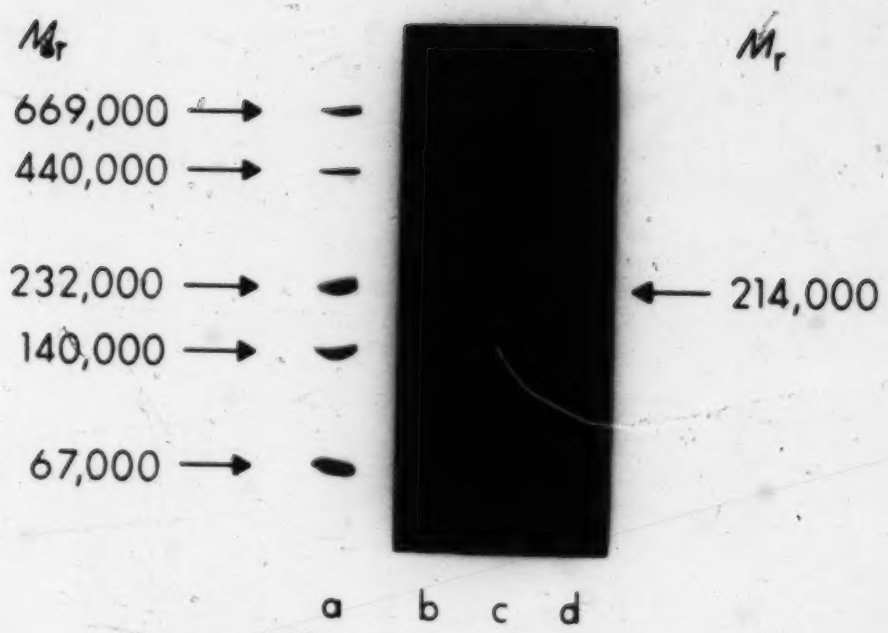


Plate 1. Gradient gel electrophoretogram of purified alkaline phosphatase and of the enzyme released from plasma membranes by proteases (5.0 mg/ml). a, protein markers; b, purified liver alkaline phosphatase; enzyme released by bromelain (c) and by ficin (d).

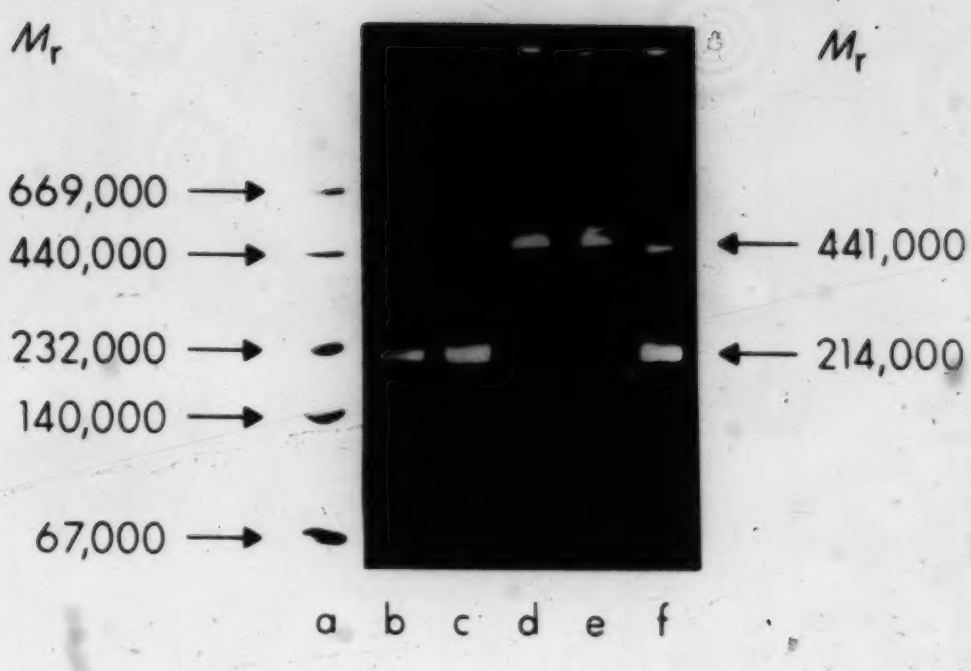


Plate 2. Gradient gel electrophoretogram of alkaline phosphatase released from plasma membranes by PI-PLC and the detergent-solubilized enzyme treated with PI-PLC. a, protein markers; b, purified liver alkaline phosphatase; c, alkaline phosphatase released from membranes by PI-PLC. d--f, NP40-solubilized alkaline phosphatase: d, control, incubated without PI-PLC; e, incubated with PI-PLC (5.0 μg/ml); f, incubated with PI-PLC (57.4 μg/ml).

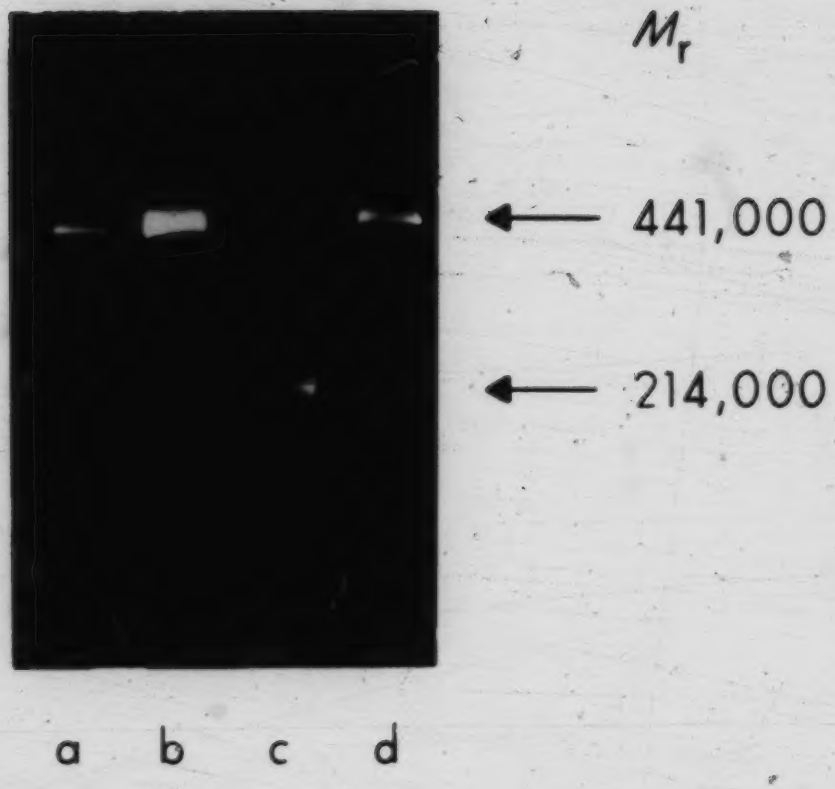


Plate 3. Gradient gel electrophoretogram of detergent-solubilized alkaline phosphatase treated with subtilisin: a, control; b, subtilisin, 0.5 mg/ml; c, subtilisin, 5.0 mg/ml; d, subtilisin, 5.0 mg/ml, and 0.02% (w/v) PMSF.

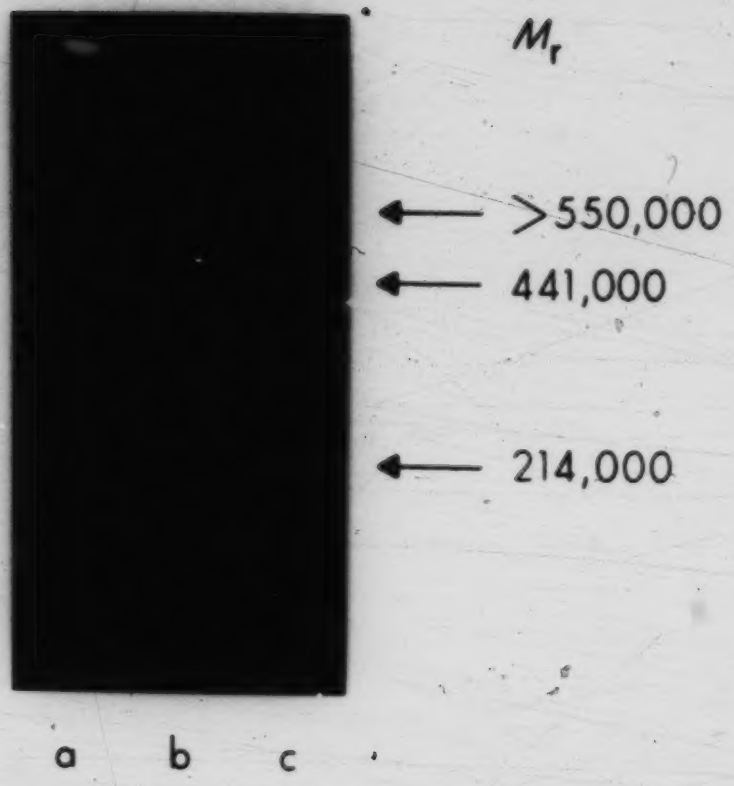


Plate 4. Gradient gel electrophoretogram of detergent-solubilized alkaline phosphatase treated with 1-butanol and PI-PLC: a, control; b, after butanol extraction; c, butanol extract treated with PI-PLC, 50.1 μg/ml.

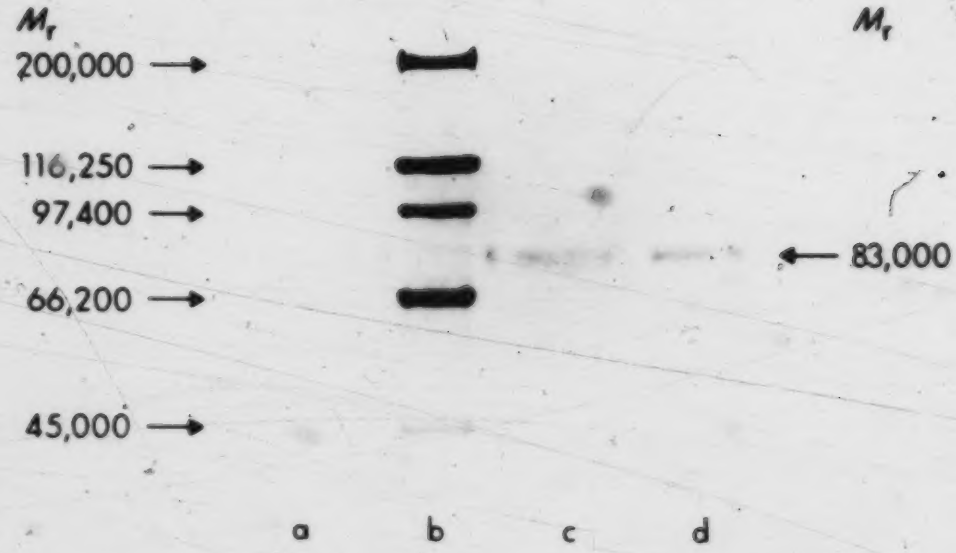


Plate 5. SDS gel electrophoretogram of purified human liver alkaline phosphatases: a, PI-PLC treated tetrameric enzyme; b, molecular weight markers; c, tetrameric enzyme; d, dimeric enzyme purified from a butanol extract of liver homogenate carried out at pH 6.6.

line phosphatase released from plasma membranes by PI-PLC or ficin did not adsorb to the hydrophobic column (Figure 7). However, the butanol-extracted form of the enzyme did, and could be eluted by a 10% (v/v) Triton solution; PI-PLC treatment of the butanol extract converted the alkaline phosphatase into a form that did not adsorb to the hydrophobic column (Figure 7). Alkaline phosphatase purified from a butanol extract of a liver homogenate carried out at pH 6.6 was also unable to adsorb to the hydrophobic column (Figure 8). However, enzyme solubilized from plasma membranes by non-ionic detergent did adsorb, and could be eluted by the addition of a 10% (v/v) Triton solution; PI-PLC treatment of the Triton-solubilized enzyme converted it into a form that did not adsorb to the column (Figure 8).

Alkaline phosphatase extracted from plasma membranes by butanol incorporated into phosphatidylcholine liposomes, with maximum incorporation requiring 12 h or more (Figure 9). Enzyme released from plasma membranes by PI-PLC or purified from a butanol extract of liver homogenate carried out at pH 6.6 did not incorporate (Figure 9). Attempts to incorporate detergent-solubilized enzyme into liposomes were unsuccessful because the liposomes were destroyed by the concentration of detergent required to solubilize the enzyme. However, butanol extraction of the detergent-solubilized enzyme stripped the detergent off the enzyme and allowed it to incorporate into liposomes (results not shown).

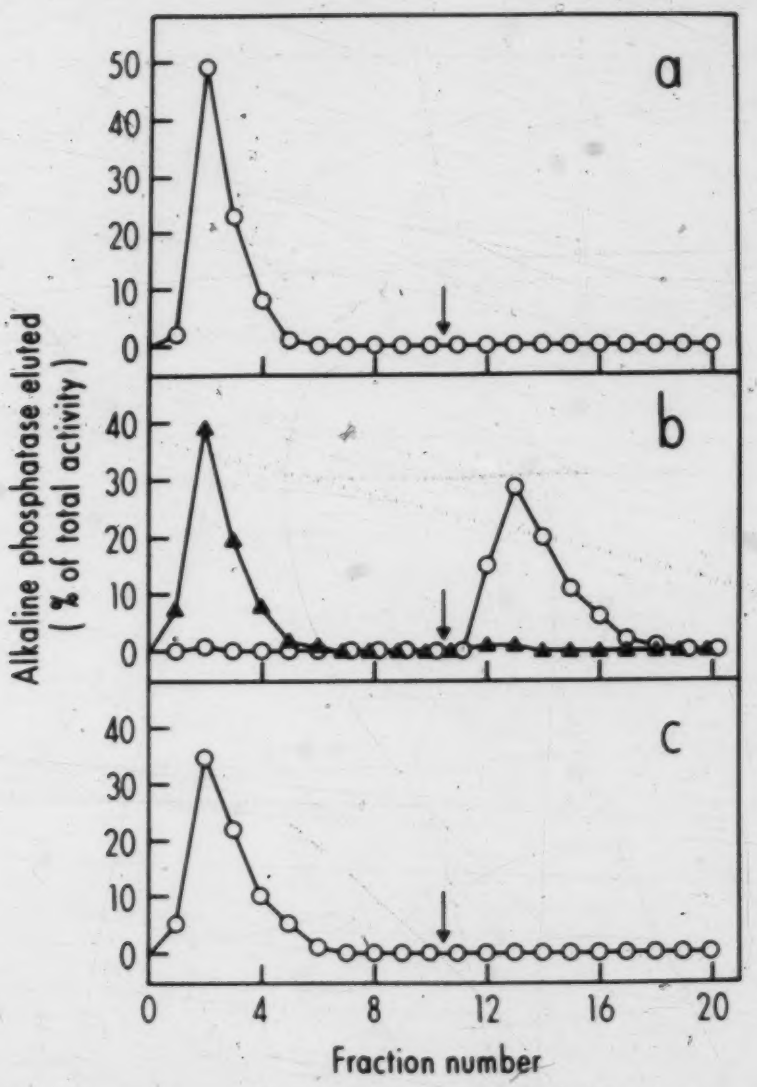


Figure 7. Elution profiles of different forms of alkaline phosphatase chromatographed on octyl-Sepharose CL-4B. The arrow indicates the application of Triton X-100 (10% (v/v)). a, alkaline phosphatase released from plasma membranes by PI-PLC; b, alkaline phosphatase extracted from plasma membranes by butanol at pH 7.6 subsequently treated with (▲) or without (○) PI-PLC; c, alkaline phosphatase released from plasma membranes by ficin.

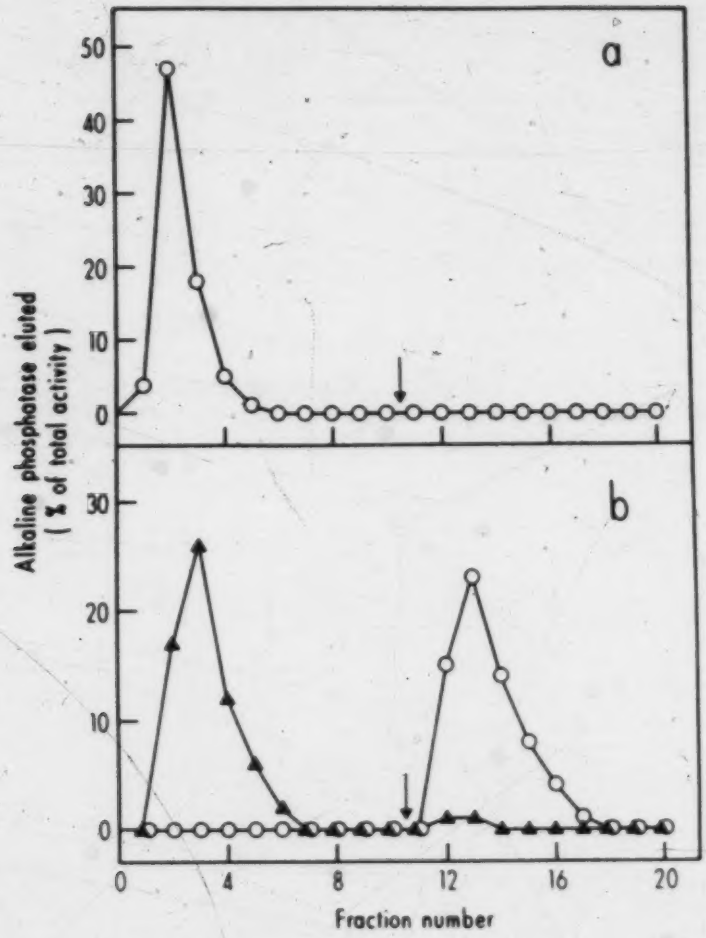


Figure 8. Elution profiles of different forms of alkaline phosphatase chromatographed on octyl-Sepharose CL-4B. The arrow indicates the application of Triton X-100 (10% (v/v)). a, alkaline phosphatase purified from a butanol extract of a homogenate of liver tissue carried out at pH 6.6; b, alkaline phosphatase solubilized from plasma membranes by Triton X-100 subsequently treated with (▲) or without (○) PI-PLC.

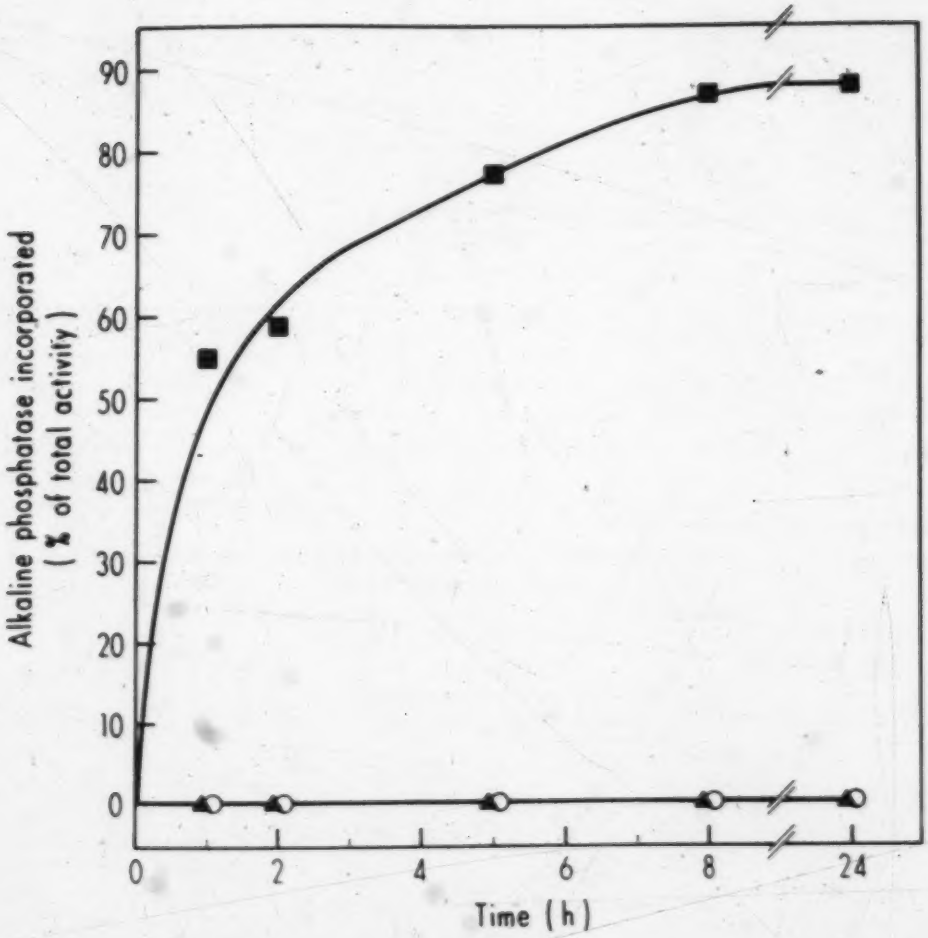


Figure 9. Incorporation of various forms of alkaline phosphatase into preformed phosphatidylcholine liposomes: ■, enzyme extracted from plasma membranes with butanol at pH 7.6; ▲, enzyme released from plasma membranes by PI-PLC; ○, enzyme purified from a butanol extract of a homogenate of liver tissue carried out at pH 6.6.

DISCUSSION

Ficin and bromelain are relatively non-specific in their choice of cleavage sites on the polypeptide chains of proteins (123). The ability of bromelain and ficin to solubilize membrane-bound alkaline phosphatase, and their lack of ability to efficiently or effectively convert NP40-solubilized alkaline phosphatase to dimers is probably due to the detergent affecting the activity or specificity of these proteases. Subtilisin Carlsberg released the membrane-bound alkaline phosphatase poorly. This protease is also non-specific in its choice of cleavage sites but is known to work well in the presence of detergent (124). It was used to convert NP40-solubilized alkaline phosphatase to dimers. Subtilisin has been previously shown to cleave a small (2 kDa) portion of the C-terminus from human placental alkaline phosphatase (125) and bromelain has also been shown to cleave a similar-sized segment from this enzyme (126).

We believe that conversion of the various forms of alkaline phosphatase from human liver occurs in a similar manner to that proposed for the membrane-bound acetylcholinesterase of Torpedo electric organ (127). This enzyme, like the alkaline phosphatase from human liver, is released from the membrane by both PI-PLC or proteolysis; however, acetylcholinesterase is a dimer in the membrane and when solubilized by detergent. This dimeric structure is maintained after treatment of the membrane-bound or detergent-solubilized forms with PI-PLC or Proteinase K. The native form of

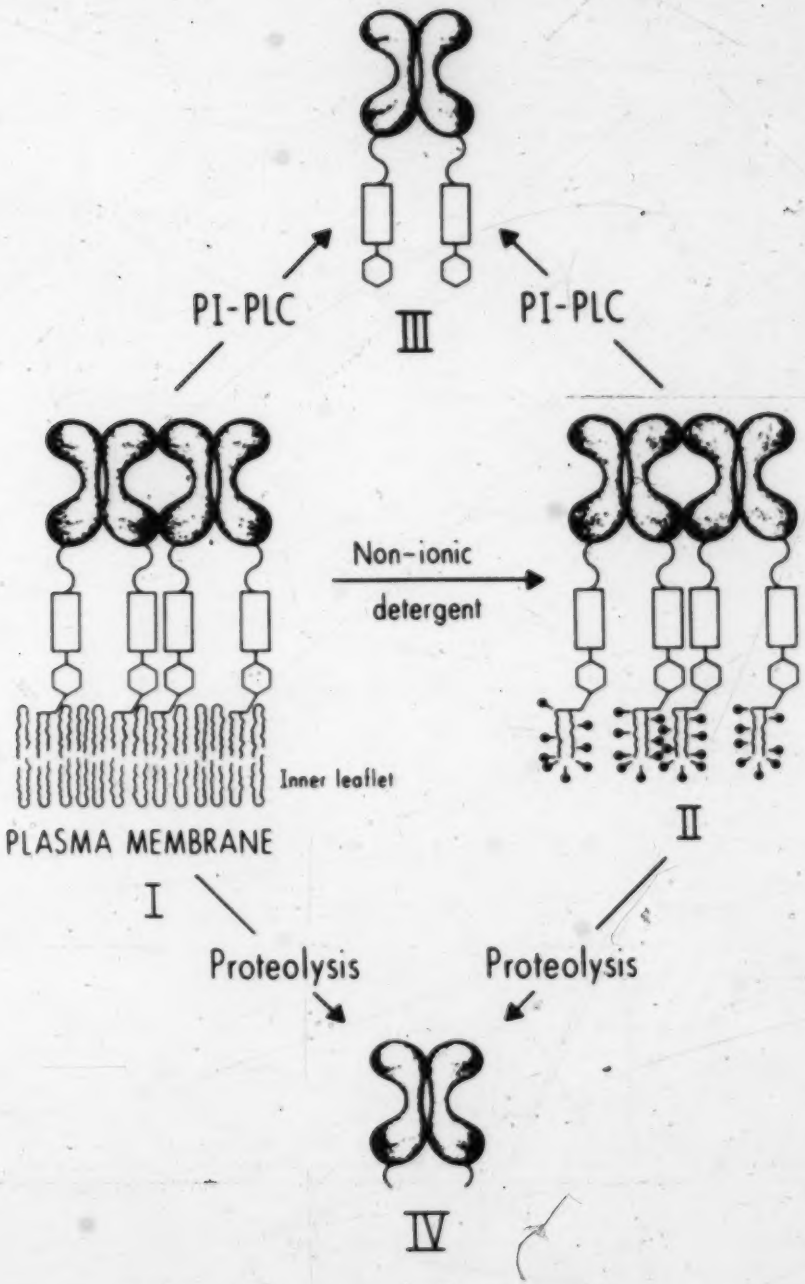
the alkaline phosphatase from human liver, however, has the $\underline{M}_r$ of a tetramer (441,000) but becomes 214,000 when treated with PI-PLC or proteases. This high $\underline{M}_r$ species is unlikely to be due to a detergent micelle since gradient gel electrophoresis is essentially an equilibrium technique and after 18 h of applied potential there should be little detergent associated with the protein. The tetramer-like enzyme, when purified to homogeneity, was composed of a single polypeptide with a subunit $\underline{M}_r$ of 83,000. Enzyme purified from a butanol extract of liver homogenate done at pH 6.6, or treatment of the purified tetrameric enzyme with PI-PLC, resulted in a dimeric form of the enzyme that had this same subunit $\underline{M}_r$. We believe the high $\underline{M}_r$ species is a tetramer composed of subunits of the same molecular mass and that cleavage of the hydrophobic membrane anchor from the polypeptides results in the formation of dimeric enzyme. Forms of the enzyme that lack the PI anchor (protease treated, PI-PLC treated, or extracted by butanol at pH 6.6) were unable to adsorb to a hydrophobic column and/or incorporate into liposomes, while forms of the enzyme having the membrane anchor (detergent-solubilized or extracted by butanol at pH 8.5) were able to incorporate into liposomes and/or adsorb to a hydrophobic column. The hydrophobic and hydrophilic forms of the enzyme obtained through butanol extractions of liver tissue done at slightly alkaline and slightly acidic pH values, respectively, may be due to an endogenous,

butanol-stimulated enzyme activity that could be a phospholipase (73,74,76) (see Chapter Four).

In the structural model we propose (Figure 10), both the membrane-bound tetrameric alkaline phosphatase and the PI molecules that are the attachment site for the enzyme are on the outer surface of the plasma membrane lipid bilayer. This location of the alkaline phosphatase and PI is based on the finding that alkaline phosphatase, 5'-nucleotidase and acetylcholinesterase from certain mammalian sources are released from intact cells by bacterial PI-PLCs without cell lysis (17,39,128,129). The attachment of the alkaline phosphatase to the PI is probably at the carboxy terminus, in view of the association of myo-inositol with the carboxy terminus of other PI-PLC releasable proteins such as mouse Thy-1 and trypanosome VSG (22,49). We presume that only one PI is attached per alkaline phosphatase monomer, in view of the almost equal ratio of myo-inositol with each subunit in Torpedo acetylcholinesterase or human placental alkaline phosphatase (67,130).

The amino acid sequence of human liver alkaline phosphatase, obtained by isolation and characterization of the cDNA corresponding to this enzyme, includes a stretch of hydrophobic amino acids at the carboxy terminal region of the enzyme precursor (131). This hydrophobic span could anchor the alkaline phosphatase to the membrane. If that were the case, however, PI-PLC treatment should not be able to solubilize the membrane-bound enzyme but proteolysis could, and

Figure 10. Structures of the various forms of alkaline phosphatase, and their interconversions: PI anchors the enzyme (possibly tetrameric) to the plasma membrane (I), and its fatty acyl groups interact with non-ionic detergent (⇢) during solubilization (II). PI-PLC cleaves both the membrane-bound and the detergent-solubilized enzyme (I and II) at a site on the PI molecule between the glycerol backbone and the inositol, leaving the hydrophobic diacylglycerol moiety in the membrane of (I) or cleaving it away (II). The alkaline phosphatase is thereby released in a hydrophilic, dimeric form (III). The similar $\underline{M}_r$ of the protease and the PI-PLC solubilized enzyme indicate that the proteolytic enzymes cleave the polypeptide chain close to the amino acid responsible for the binding to the PI, creating an effect similar to that with PI-PLC release (IV). In the diagram a small peptide tail is shown attached to the glycan structure represented by the box, which in turn is attached to the inositol headgroup of the phospholipid.


PI-PLC
III
PI-PLC
Non-ionic
detergent
Inner leaflet
PLASMA MEMBRANE
I
II
Proteolysis
Proteolysis
IV

the latter would leave the hydrophobic 'tail' in the membrane. As the enzyme is solubilizable by both PI-PLC and proteolysis, the most likely thing to occur is post-translational processing in which the carboxy terminus (containing a span of hydrophobic amino acids) of the enzyme precursor is proteolytically detached and the PI is attached, directly or indirectly, to an amino acid near or at the newly formed carboxy terminus. Thus, the mature enzyme would be anchored to the membrane by PI and not by a membrane-spanning stretch of hydrophobic amino acids. This type of post-translational modification probably occurs in the trypanosome membrane VSG (132); also, cDNA analysis of rat Thy-1 (133), another PI-PLC releasable membrane-bound protein, has shown 32 extra amino acids at the carboxy terminus (of which 20 are hydrophobic) that are absent from the mature protein (49).

As the human liver alkaline phosphatase is attached to the PI, solubilization by a detergent requires interaction of the hydrophobic portion of the detergent's molecules with the PI's fatty acyl chains. This treatment, however, with non-ionic detergent can do little to disrupt the forces holding two pairs of dimers together, and the enzyme thus solubilized is tetrameric. When tetrameric alkaline phosphatase is treated with PI-PLC or proteolytic enzymes, conversion to dimers occurs. Since the interaction with PI is needed for membrane-binding or detergent-solubilization, the dimeric forms of the alkaline phosphatase would be

water-soluble since they should not have the fatty acyl chains of the PI associated with them. The inability of alkaline phosphatase solubilized from membranes by either PI-PLC or a protease to adsorb to a hydrophobic column is also evidence that the enzyme is in a hydrophilic form that lacks the fatty acyl chains of the PI membrane anchor. Although we do not have definitive evidence that human liver alkaline phosphatase is present as a tetramer on the plasma membrane itself, it is a possibility since we have shown that mouse osteosarcoma alkaline phosphatase has a tetrameric structure upon release from the membranes by PI-PLC (unpublished). As yet we have been unable by cross-linking experiments to directly demonstrate the existence of a tetramer in the plasma membrane of human liver cells.

Treatment of the plasma membranes with butanol at a slightly alkaline pH will extract the lipids of the bilayer but leave the PI attached to the alkaline phosphatase (present in the aqueous phase of the extract). The high affinity of the fatty acyl chains of this PI for those of the PI on the enzyme's other molecules induces the formation of aggregates of alkaline phosphatase, treatment of which with PI-PLC frees the enzyme from the attached PI and renders it dimeric in M_r. Similarly, alkaline phosphatase solubilized by detergent and then cleansed of it by butanol extraction, clumps in aggregates of high M_r that PI-PLC can convert to dimers. Human liver alkaline phosphatase behaves similarly to the enzyme from pig kidney in that the porcine

enzyme formed aggregates upon butanol extraction that were excluded on Sepharose 6B. These aggregates could be partially broken down by PI-PLC (134). The ability of alkaline phosphatase extracted from plasma membranes by butanol at pH 7.6 or solubilized by non-ionic detergent to adsorb to a hydrophobic column, but the inability of butanol-extracted or detergent-solubilized alkaline phosphatase treated with PI-PLC to do so, points to the fact that the major hydrophobic portion of the enzyme must be the fatty acyl chains of the attached PI.

Thus it seems likely that the major force between pairs of dimers maintaining the tetrameric structure resides in the PI molecules that are attached to the enzyme: when the PI is lost, as when the alkaline phosphatase is released from membranes or from detergent--lipid complexes by PI-PLC or proteolysis, the enzyme takes on a dimeric structure. Physiologically, a tetrameric structure of human liver alkaline phosphatase and its binding to PI may be necessary in carrying out its function. Little is known of this function, although the membrane-bound form of the enzyme has been shown to dephosphorylate certain human liver plasma membrane proteins (107,108).

CHAPTER FOUR

CONVERSION OF ALKALINE PHOSPHATASE FROM A HYDROPHOBIC TO A HYDROPHILIC FORM BY A BUTANOL-STIMULATED ENZYME ACTIVITY ENDOGENOUS TO HUMAN LIVER

A. INTRODUCTION

The existence of a butanol-stimulated phospholipase that can convert alkaline phosphatase from a hydrophobic to a hydrophilic form has been discussed previously (see Chapter One, Section I). This chapter describes experiments designed to characterize this activity that is endogenous to human liver plasma membranes.

B. MATERIALS AND METHODS

1. Chemicals

Phospholipase C (Bacillus cereus) type III, p-nitrophenylphosphate, Tris, MOPS (3-[N-morpholino]propanesulfonic acid), MES (2[N-morpholino]ethanesulfonic acid), PMSF, pepstatin A, and naphthol AS-MX phosphate were purchased from Sigma (St Louis, MO), 1-butanol from BDH (Darmstadt, West Germany), ethylanimoethanol from Aldrich (Milwaukee, WI), molecular-mass markers for electrophoresis and octyl-Sepharose CL-4B from Pharmacia (Uppsala, Sweden), and 2.5--27% polyacrylamide gradient gels in rods from Isolab (Akron, OH). All other chemicals were reagent grade (Fisher Scientific, Fairlawn, NJ).

2. Plasma Membrane Preparation, Enzyme Assays, and Molecular Mass Determinations

Plasma membrane preparation and alkaline phosphatase assays are described in Chapter Two, Section B, and the molecular mass determinations are described in Chapter Three, Section B.

3. Effect of Varying pH Values During Butanol Extractions

An aliquot of human liver plasma membranes was suspended in each of the following buffers: 50 mmol/l MES, pH 6.0; 50 mmol/l MOPS, pH 7.0; and 50 mmol/l Tris, pH 8.0. Each sample was extracted with butanol (Chapter Three, Section B), and the aqueous layer applied to a column of octyl-Sepharose (Chapter Three, Section B). A portion of the aqueous layer was also subjected to gradient gel electrophoresis (Chapter Three, Section B). For the samples run on the gels, corresponding negative controls (i.e. membranes at the sample pH, but shaken without the butanol present) were also run.

4. Time Course for the Conversion of Alkaline Phosphatase From a Hydrophobic to a Hydrophilic Form

Human liver plasma membranes suspended in 50 mmol/l MES, pH 6.0, were extracted with 1-butanol at room temperature for varying intervals of time. Each extract was then assayed by an octyl-Sepharose procedure to determine the conversion of the alkaline phosphatase from a hydrophobic to a hydrophilic form. The octyl-Sepharose assay was done as follows: 50 µl of packed octyl-Sepharose beads were mixed with

100 µl of butanol extract. This was mixed for 5 min at room temperature on a rocking mixer and spun at 10,000xg for 10 min. The supernatant, containing the hydrophilic form of alkaline phosphatase, was assayed and the activity expressed as a percent of the total activity of the sample (total activity is the activity present before adsorption to octyl-Sepharose).

5. The Effect of Protease Inhibitors on the Conversion of Alkaline Phosphatase to a Hydrophilic Form During Butanol Extraction

Human liver plasma membranes were suspended in 50 mmol/l MES, pH 6.0. This was divided into three aliquots. To one was added PMSF to a final concentration of 0.02% (w/v) and to another pepstatin A (50 µg/ml). The negative control contained no protease inhibitors. The samples were allowed to stand for 15 min before the addition of butanol in order to allow the PMSF to react (the PMSF is hydrophobic and may partition into the butanol phase). The membranes were then extracted with 1-butanol for 1 h at room temperature and the percent hydrophilic alkaline phosphatase in each aliquot was subsequently determined by using the octyl-Sepharose assay described previously.

6. The Effect of Cations on the Conversion of Alkaline Phosphatase to a Hydrophilic Form

Aliquots of human liver plasma membranes, suspended in 50 mmol/l MES, pH 6.0, had added to them cations (Mg^{2+},

Mn^{2+}, or Ca^{2+}) to a final concentration of 2 mmol/l. The membranes were extracted with 1-butanol for 1 h at room temperature and then the percent hydrophilic alkaline phosphatase in each aliquot was determined by using the octyl-Sepharose assay described previously.

C. RESULTS

A low pH and 1-butanol promoted the conversion of alkaline phosphatase from an aggregated (predominantly tetrameric) to a dimeric form (Plate 6). The enzyme not only underwent a change in its molecular mass but also was converted from a hydrophobic to a hydrophilic form (Figure 11). This conversion was shown to be a time-dependent process (Figure 12). The presence of divalent cations did not significantly stimulate this conversion and only Mn^{2+} was inhibitory (Figure 13). The conversion of alkaline phosphatase to a hydrophilic form was 48% in the presence of PMSF, 51% in the presence of pepstatin A, and 48% in the presence of no protease inhibitors.

D. DISCUSSION

The data suggest that an enzyme endogenous to human liver plasma membranes converts alkaline phosphatase from a hydrophobic to a hydrophilic form during extraction with 1-butanol. A similar type of activity has been observed in other alkaline phosphatase-containing tissues, both human and non-human (72--76). The conversion in human liver plasma

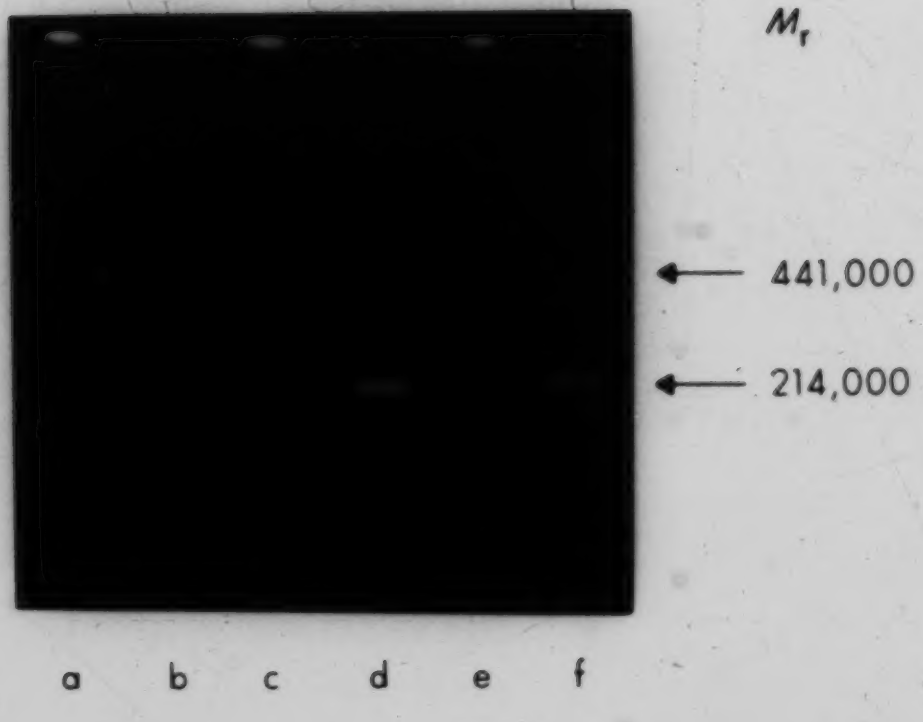


Plate 6. Gradient gel electrophoretogram of membrane-bound alkaline phosphatase treated with butanol at different pH values. a, no butanol, pH 8.0; b, butanol extract, pH 8.0; c, no butanol, pH 7.0; d, butanol extract, pH 7.0; e, no butanol, pH 6.0; f, butanol extract, pH 6.0.

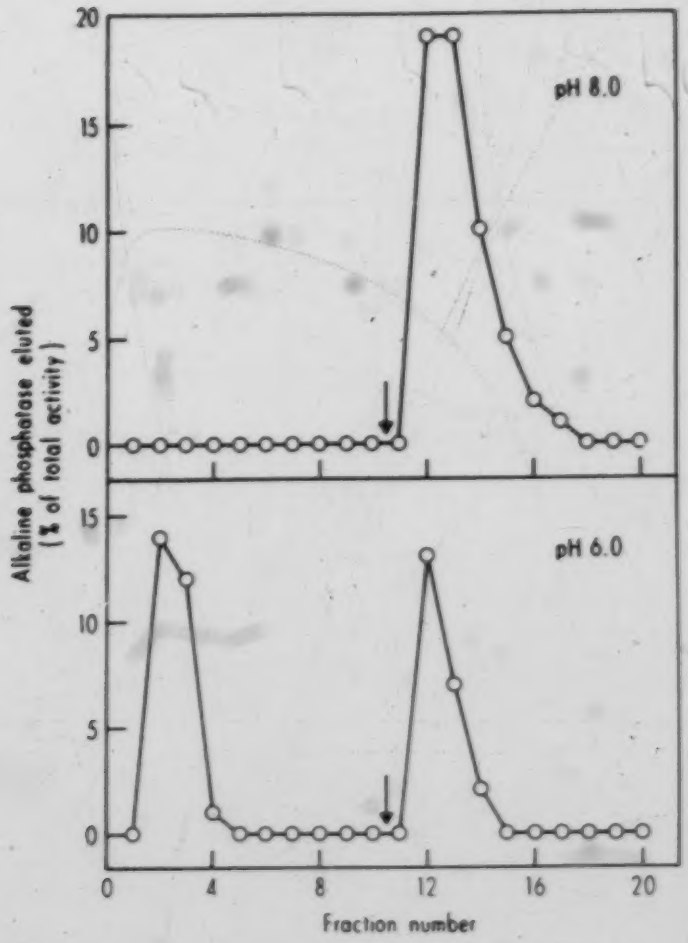


Figure 11. Elution profiles of different forms of alkaline phosphatase chromatographed on octyl-Sepharose CL-4B. The arrow indicates the application of Triton X-100 (10% (v/v)): top, membrane-bound alkaline phosphatase treated with butanol at pH 8.0; bottom, membrane-bound alkaline phosphatase treated with butanol at pH 6.0.

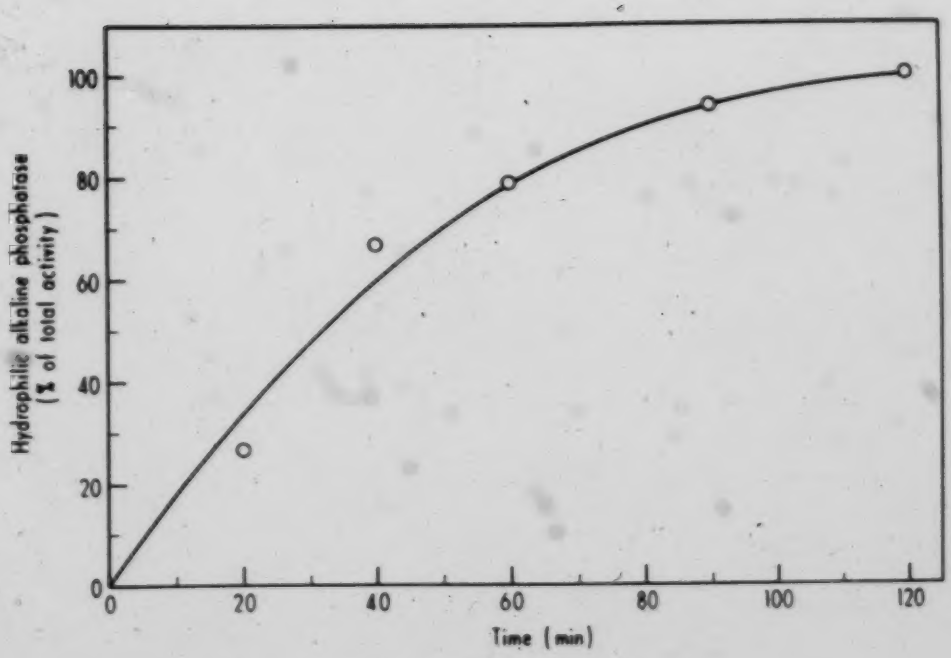


Figure 12. Conversion of membrane-bound alkaline phosphatase from a hydrophobic to a hydrophilic form by treatment with 1-butanol at pH 6.0.

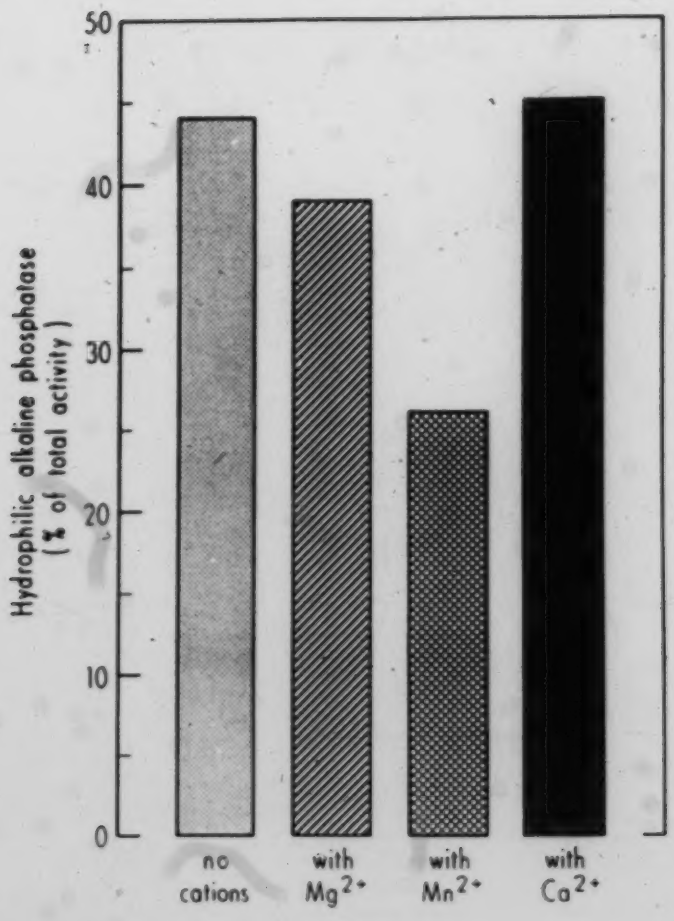


Figure 13. Effect of divalent cations at 2 mmol/l on the conversion of membrane-bound alkaline phosphatase from a hydrophobic to a hydrophilic form by treatment with 1-butanol at pH 6.0.

membranes was pH-dependent, occurring optimally at a slightly acidic pH, and was inhibited by Mn^{2+} . Although these results do not as yet rule out a non-enzymatic process, they do point to the possibility of an enzyme mediating the conversion of the alkaline phosphatase from a hydrophobic to a hydrophilic form. The protease inhibitors PMSF and pepstatin A did not inhibit this process, so it is unlikely that the conversion is due to a protease. Based on results reported in earlier chapters of this thesis, it is likely that the membrane-associated activity that converted the alkaline phosphatase from a hydrophobic to a hydrophilic form is a phospholipase of the C or D type.

CHAPTER FIVE

GENERAL CONCLUSIONS

The major purpose of this study was to determine the nature of the membrane anchor of human liver alkaline phosphatase and the effect of this anchor on the quaternary structure of this enzyme. What will be discussed is how this may relate to the *in vivo* function of the enzyme.

The possible tetrameric structure of the alkaline phosphatase when it is attached to the plasma membrane may be essential for it to perform its physiological role(s). Since it is anchored via PI, lateral diffusion could be significantly faster than if it were anchored by a hydrophobic polypeptide, thus allowing the enzyme quicker access to its substrate(s). Even though *in vitro* kinetic studies have so far shown no major differences between the catalytic activities of the membrane-bound and the dimeric, soluble form of this enzyme for any particular substrate (87), that doesn't imply that the same must hold true under *in vivo* conditions. Physiologically, major differences in substrate affinities may be helpful under the following circumstance. If a substrate could be utilized only by the tetrameric form of the alkaline phosphatase, then release of the enzyme by an endogenous phospholipase would form a dimeric enzyme that may have a greatly reduced affinity for the substrate in question. This endogenous phospholipase may be what is responsible for the butanol-stimulated *in vitro* conversion of the

alkaline phosphatase from a membrane-bound to a soluble form. Thus, a change in quaternary structure brought about by release of the enzyme from the plasma membrane could effectively be used to down-regulate the enzyme under the appropriate physiological circumstances.

Even though the *in vivo* function(s) of human liver alkaline phosphatase is unknown as of this time, a knowledge of the nature of the membrane anchor and of the changes in enzyme quaternary structure caused by the presence or absence of this anchor should help to contribute to a full understanding of this enzyme and how its catalytic activity may be regulated *in vivo*.

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PUBLICATIONS ARISING FROM THIS THESIS

Hawrylak, K. and Stinson, R.A. (1987) Tetrameric alkaline phosphatase from human liver is converted to dimers by phosphatidylinositol phospholipase C. FEBS Lett. 212, 289-291.

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